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Dimethyl fumarate, a small molecule drug for psoriasis, inhibits Nuclear Factor-κB and reduces myocardial infarct size in rats

Silvia Meili-Butz [a,*], Thomas Niermann [a], Elizaveta Fasler-Kan [b], Vania Barbosa [a], Nicole Butz [c], Dietlinde John [a], Marijke Brink [a], Peter T. Buser [a], Christian E. Zaugg [a]

[a] *University Hospital Basel, Department of Research, Cardiobiology Laboratories, Switzerland*
[b] *Pediatric Surgical Oncology, Hebelstrasse 20, 4031 Basel, Switzerland*
[c] *Vascular Biology, Hebelstrasse 20, 4031 Basel, Switzerland*

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Abstract

Persistent Nuclear Factor-κB (NF-κB) activation is hypothesized to contribute to myocardial injuries following ischemia–reperfusion. Because inhibition or control of NF-κB signaling in the heart probably confers cardioprotection, we determined the potency of the NF-κB inhibitor dimethyl fumarate (DMF) in cardiovascular cells, and determined whether administration of DMF translates into beneficial effects in an animal model of myocardial infarction. In rat heart endothelial cells (RHEC), we analysed inhibitory effects of DMF on NF-κB using shift assay and immunohistofluorescence. In *in vivo* experiments, male Sprague Dawley rats undergoing left coronary artery occlusion for 45 min received either DMF (10 mg/kg body weight) or vehicle 90 min before ischemia as well as immediately before ischemia. After 120 min of reperfusion, the hearts were stained with phthalocyanine blue dye and triphenyltetrazolium chloride. Additionally, acute hemodynamic and electrophysiologic effects of DMF were determined in dose–response experiments in isolated perfused rat hearts. DMF inhibited TNF-α-induced nuclear entry of NF-κB in RHEC. In *in vivo* experiments, myocardial infarct size was significantly smaller in rats that had received DMF (20.7%±9.7% in % of risk area; $n=17$) than in control rats (28.2%±6.2%; $n=15$). Dose–response experiments in isolated perfused rat hearts excluded acute hemodynamic or electrophysiologic effects as mechanisms for the effects of DMF. DMF inhibits nuclear entry of NF-κB in RHEC and reduces myocardial infarct size after ischemia and reperfusion in rats *in vivo*. There was no indication that the beneficial effects of DMF were due to acute hemodynamic or electrophysiologic influences.

Keywords: Myocardial infarction; Ischemia/reperfusion; NF-κB; Cardioprotection; Dimethyl fumarate

1. Introduction

Recent evidence suggests that myocardial ischemia/reperfusion correlates with an acute increase of proinflammatory molecules including reactive oxygen species (Bolli et al., 2002), and cytokines (Loewe et al., 2002; Misra et al., 2003), which are known activators of Nuclear Factor-κB (NF-κB). Accordingly, activation of NF-κB has been reported in post-ischemic reperfusion (Kis et al., 2003) and in myocardial infarction, which possibly corresponds to a primary activation by reactive oxygen intermediates and a secondary activation by proinflammatory cytokines (Chandrasekar and Freeman, 1997). NF-κB regulates the coordinated expression of a diverse group of genes including cytokines, metalloproteinases and adhesion molecules that appear to mediate the development of myocardial damage following ischemia and reperfusion (Morishita et al., 1997; Valen et al., 2001). In agreement with the presumed detrimental role of cardiac NF-κB in reperfusion and infarction, recent studies have provided substantial evidence that blockade of NF-κB signaling ameliorates its deteriorating effects. Cardioprotection via influencing NF-κB has been achieved through

* Corresponding author. University Hospital Basel, Department of Research, Lab 319, Hebelstr. 20, 4031 Basel, Switzerland. Tel.: +41 61 2652345; fax: +41 61 2652350.
E-mail address: Silvia.Meili@unibas.ch (S. Meili-Butz).

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various pathways and in diverse experimental settings (Li et al., 2000; Morishita et al., 1997; Pye et al., 2003; Sawa et al., 1997; Yeh et al., 2005; Zingarelli et al., 2002). Furthermore, genetic models in which inducible NF-κB activity is repressed by myocardial expression of NF-κB inhibitor proteins, inhibitor κB (IκB) and A20, have further highlighted the relationship between NF-κB and different cardiac pathophysiological processes (Brown et al., 2005; Cook et al., 2003; Squadrito et al., 2003).

Taken together, data from various approaches provide a convincing basis to consider targeted NF-κB inhibition in clinical treatment. Dimethyl fumarate (DMF) is an active ingredient of the oral antipsoriatic drug Fumaderm® (Fumapharm, Switzerland) and it has been shown to inhibit Tumor necrosis factor-α (TNF-α)-induced activation of NF-κB in endothelial cells *in vitro*. In human endothelial cells, DMF inhibits TNF-α-induced tissue factor mRNA transcription and protein expression. DMF inhibits NF-κB induced gene transcription at the level of nuclear entry of p65 after its release from IκB (Loewe et al., 2002). Thus, DMF might also inhibit NF-κB activity in cardiovascular cells and thus reduce myocardial infarction following ischemia and reperfusion. In the present study we tested possible effects of DMF on cardiovascular cells, and we determined whether these effects translate into beneficial effects in an animal model of myocardial infarction.

2. Materials and methods

2.1. Cell culture and reagents

We isolated rat heart endothelial cells (RHEC) from hearts of adult male Sprague Dawley rats by enzymatic digestion using a method modified from Belke et al. (2002) and separated the RHEC from other cardiac cell types by centrifugation and adhesion to plastic surfaces. RHEC were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Basel, Switzerland) containing 10% fetal bovine serum (Oxoid AG, Basel, Switzerland) at 37 °C in an atmosphere of 5% CO_2. The RHEC used in this study showed typical cobblestone appearance and more than 95% of cells incorporated dil-acetylated low-density lipoprotein (Biocoba AG, Switzerland) or isolectin (I-21411, Molecular Probes) with negative immunostaining for α-smooth muscle actin (C6198, Sigma). We used cells from passages 1 to 4 for experiments and treated them with recombinant rat TNF-α (rrTNF-α, R&D Systems, Abingdon, United Kingdom), lipopolysaccharide (LPS, Sigma, Buchs, Switzerland), DMF (Fumapharm AG, Lucerne, Switzerland), methyl hydrogen fumarate (MHF, Fumapharm AG), Pyrrolidinedithiocarbamic acid (PDTC, Sigma) as described in the figure legends; dimethyl sulfoxide (DMSO, Sigma) was used as the solvent control for DMF.

2.2. Nuclear protein preparation

After discarding the cell culture medium we washed the cells with 5 ml ice cold PBS. We scraped the cells into 5 ml fresh PBS and collected them by centrifugation for 5 min at 3500 g in a Labfuge 400 (Heraeus Instruments, Kendro Laboratory Products, Zurich, Switzerland). By vortexing we suspended the cell pellet in 1 ml lysis buffer (20 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, 1 mM dithiothreitol) and incubated it on ice for 10 min. We collected nuclei by centrifugation for 5 min at 3500 g and resuspended them in a threefold volume of extraction buffer (20 mM HEPES [pH 7.9], 400 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 1× Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Reinach Switzerland)). With gentle vortexing, we incubated nuclear suspension at 4 °C, for 20 min. We centrifuged the suspension for 30 min at 15,339 g in an Eppendorf 5415 centrifuge and removed the top layer containing the nuclear proteins from the cell debris. The protein concentration we determined using the Bradford reagent (Bio-Rad, Reinach, Switzerland).

2.3. Electrophoretic mobility shift assay (EMSA)

We performed EMSA using complementary DNA oligonucleotides (Microsynth, Balgach, Switzerland) comprising the consensus sequences for NF-κB (5′-ACT TGAGGGGATTTCCCAGGC-3′ and 5′-GCCTGGGAAATCCCCTCAAGT-3′). Oligonucleotides were end-labeled with [γ^{-32}P] ATP (Amersham, Otelfingen Switzerland) using T4-polynucleotide kinase (Promega, Basel, Switzerland). For EMSA, we incubated 10 μg nuclear extract in 15 μl reaction volume supplemented with 2 μg poly [d(I–C)] (Roche Molecular Biochemicals, Reinach, Switzerland), 2 mmol/l dithiothreitol, 10 mmol/l HEPES pH 8.4 at 30 °C for 30 min. Then we added 10 fmol of [γ^{-32}P]-end-labeled oligonucleotide containing the NF-κB binding site and incubated the mixture at room temperature for another 30 min. In competition assays, we used 50-fold excess unlabeled oligonucleotide. For supershift assays, we added 1 μg of anti-p50 and anti-p65 antibodies (Santa Cruz) to the nuclear protein solution 20 min before addition of the labelled oligonucleotide. The DNA–protein complexes were electrophoresed in 5% polyacrylamide gels, dried and autoradiographed.

2.4. Immunofluorescence assay

We cultured the RHEC in cover slips in 24 well plates. Sixteen hours before the start of experiments, we starved the cells. For activation of the NF-κB pathway we treated the cells with rrTNF-α (0.1 ng) or left them untreated (negative control). For blocking experiments after starvation we incubated the cells with DMF (84 μM), MHF (84 μM) (Loewe et al., 2002) or PDTC (80 μM) (Iseki et al., 2000) for 60 min. After washing with PBS, we stimulated the cells with rrTNF-α (0.1 ng) for 20 min. We fixed the cells in 4% paraformaldehyde for 30 min followed by permeabilisation in Triton X-100 0.2% for 5 min. Finally, we used anti-p65 antibody from Santa Cruz as primary antibodies and goat anti-rabbit (Alexa Fluor 555, Molecular Probes Inc., Eugene, USA) as secondary antibodies. For staining of nuclei we added DAPI 0.001% (Sigma).

2.5. In vivo experiments

2.5.1. Animals

We performed all experiments in male Sprague Dawley rats from Iffa credo (L'Arbresle, France). Furthermore, all experiments conformed to the rules of the Swiss Federal Act on Animal Protection (1998) and to NIH Guidelines, and were approved by the Veterinary Department of Basel (Switzerland).

2.5.2. Animal model of acute myocardial ischemia and reperfusion

We used a rat model of reversible myocardial ischemia and reperfusion (Barbosa et al., 1996). Accordingly, we anesthetized male Sprague Dawley rats weighing 200–250 g using isoflurane 1.3% (Forene®, Abbott AG, Baar, Switzerland) in O_2 (30 ml/min)/N_2O (90 ml/min). After incubation we performed a midline sternotomy and placed a reversible coronary artery snare occluder (Ti-Cron 7-0, Sherwood-Davis & Geck, St. Louis, USA) around the proximal left coronary artery. After a stabilization time of 20 min, we started the experimental protocol as described below. Throughout the experiments, a rectal thermometer monitored core body temperature of the rats and maintained the body temperature of rat at 37.0±0.1 °C.

2.5.3. Experimental protocol

In a blinded design we randomly assigned the rats to one of the three experimental groups: a) to the DMF group receiving 10 mg/kg body weight DMF (dissolved in DMSO 2% in water), or b) to the vehicle group receiving only the vehicle (DMSO 2% in water; necessary to dissolve DMF), or c) to the positive control group receiving the vehicle plus ischemic preconditioning (IPC, known to reduce infarct size) (Barbosa et al., 1996). This dose of 10 mg/kg body weight of DMF corresponded approximately to a maximally tolerated dose in man. As we could confirm in our *in vitro* experiments that DMF is the active compound (Gerdes et al., 2007; Gesser et al., 2007; Loewe et al., 2001), we performed *in vivo* experiments with DMF but not with MHF. DMF and the vehicle, respectively, we administered i.v. into tail vein as bolus injection (Diehl et al., 2001) 90 min before ischemia (under general anesthesia using isoflurane) as well as immediately before ischemia. We induced ischemic preconditioning by two times 5 min episodes ischemia (induced by left coronary artery occlusion) each followed by 5 min of reperfusion (induced by releasing the snare). To determine the effect of DMF on infarct size, we subjected all rats to a 45-minute period of ischemia by left coronary artery occlusion followed by 120 min of reperfusion, and then assessed for infarct size. We performed the experiments (except pilot experiments) pair wise, i.e. two experiments in parallel avoiding identical group assignment. The present study was planned, set up, and permitted to demonstrate a pharmacodynamic effect of DMF on myocardial infarct size in rats *in vivo*. An eventual mode of action should be investigated *in vitro*. The results from our *in vitro* experiments as well as results of other laboratories did not show any effect of MHF on NF-κB activation. Therefore, no experiment was included to exclude MHF from the mode of action of DMF.

2.5.4. Variables

2.5.4.1. Infarct size. We determined infarct size using a double staining technique with phthalocyanine blue dye (Engelhard, Iselin, USA) and 2,3,5-triphenyltetrazolium chloride (TTC, Fluka, Switzerland) as previously described (Barbosa et al., 1996). After staining, we fixed the left ventricular sections in a 4% formalin solution. An operator, blinded regarding the group assignment of the experiment, outlined the ischemic risk area (unstained by phthalocyanine blue dye) and the infarcted area in each section (unstained by TTC) on the digital images, measured them by planimetry, averaged the area from both sides of each slice, and multiplied the value by the weight of the tissue of that slice. We expressed infarct size both as a percentage of total left ventricular mass and as a percentage of the ischemic risk area.

2.5.4.2. Electrocardiogram, heart rate, and QT interval. We recorded a standard lead II electrocardiogram (ECG) according to Einthoven. The digitized ECG enabled the analysis of heart rate before, during, and after ischemia. Finally, we analysed QT intervals before ischemia. In order to correct for heart rate we used a correction formula developed by Bazett on the QT interval (observed QT interval is divided by the square root of the RR interval) and the linear regression analysis described in the Framingham Heart Study ($QTc = QT + 0.154 \times (1 - RR)$) (Brouwer et al., 2003).

2.6. Control experiments in the isolated perfused rat heart

To exclude the possibility that beneficial properties of DMF or its main metabolite, MHF were due to acute hemodynamic or electrophysiologic effects, we assessed the acute effects of DMF and MHF on heart rate, coronary flow, left ventricular developed pressure, and duration of monophasic action potentials at 90% repolarisation ($MAPD_{90\%}$) in dose–response curves in nine isolated perfused rat hearts *in vitro* according to

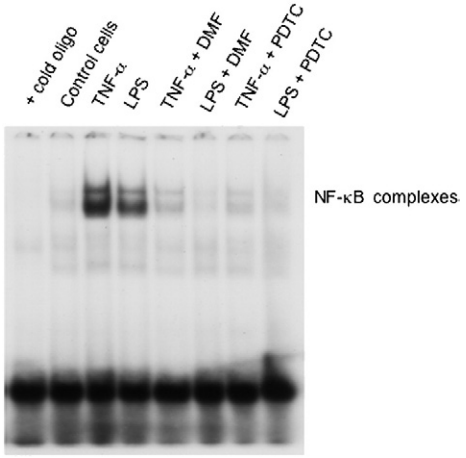

Fig. 1. Effects of DMF and PDTC on rrTNF-α- and LPS-dependent activation of NF-κB in rat heart endothelial cells. Rat heart endothelial cells were preincubated with 100 μM DMF or PDTC for 3 h and incubated with rrTNF-α 5 ng/ml or LPS 5 μg/ml for 1 h ($n=3$).

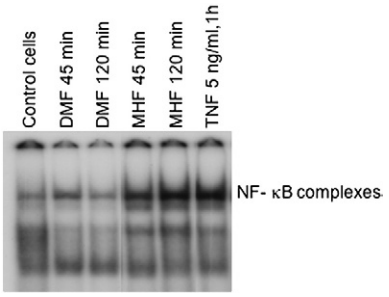

Fig. 2. Effects of DMF and MHF on rrTNF-α-dependent activation of NF-κB in rat heart endothelial cells. Rat heart endothelial cells were preincubated with 10 μM DMF or MHF for the indicated time and incubated with rrTNF-α 5 ng/ml for 1 h ($n=3$).

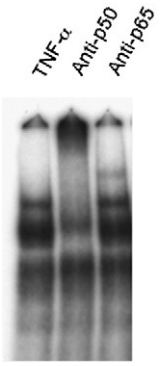

Fig. 4. Supershift experiment: Antibodies specific for the p50 (lane 2) or p65 (lane 3) subunit of NF-κB were added prior to EMSA reaction ($n=3$).

Langendorff as described previously (Butz et al., 2004). We perfused all hearts at a constant pressure of 80 mm Hg with a filtered (pore size 0.65 μM) nonrecirculating modified Krebs–Henseleit buffer containing (in mM) NaCl 117, KCl 4.3, $MgCl_2$ 1.2, $CaCl_2$ 2.0, $NaHCO_3$ 25, EDTA 0.5 and glucose 15, and albumin 10.0 mg/l at pH 7.4 saturated with 95% O_2/5% CO_2. DMF (0.1 μM to 1000 μM, $n=9$) and MHF (0.1 μM to 1000 μM, $n=9$) were perfused consecutively, the sequence of which was switched for each experiment. To dissolve DMF we used a Krebs–Henseleit buffer containing 0.004% DMSO. MHF was dissolved in the standard Krebs–Henseleit buffer. To rule out effects of the vehicle of DMF, we administered 0.004% DMSO alone prior to DMF perfusion.

2.6.1. Measurement of hemodynamic and electrophysiological variables

An inline flow probe (Transonic 2N) connected to a transit time flow meter (Transonic TTFM-SA type 700, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) measured coronary flow within the aortic canula. This inline flow probe allowed continuous and reliable recording of coronary flow throughout the experiments. We measured left ventricular pressure by a fluid-filled polyethylene catheter inserted through the left atrial appendage into the left ventricular cavity. The catheter was connected to a pressure transducer (MLT1050 Pressure transducer, AD Instruments, Castle Hill, Australia). We defined left ventricular developed pressure as the difference between systolic and diastolic values of left ventricular pressure. A pair of electrodes placed on the right appendage and apex recorded a bipolar epicardial ECG. We recorded monophasic action potentials using two contact electrode catheters (Ag–AgCl electrode, model 225, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) on the epicardium of the left ventricle and of the right ventricle. For reliable results it was important that the position of the electrodes and the contact pressure stayed the same throughout the experiments.

2.7. Evaluation and statistical analysis

We expressed infarct size as mean±S.D. after confirmation of normal distribution of the data by Shapiro–Wilk test. Comparisons of infarct size between groups were performed using analysis of

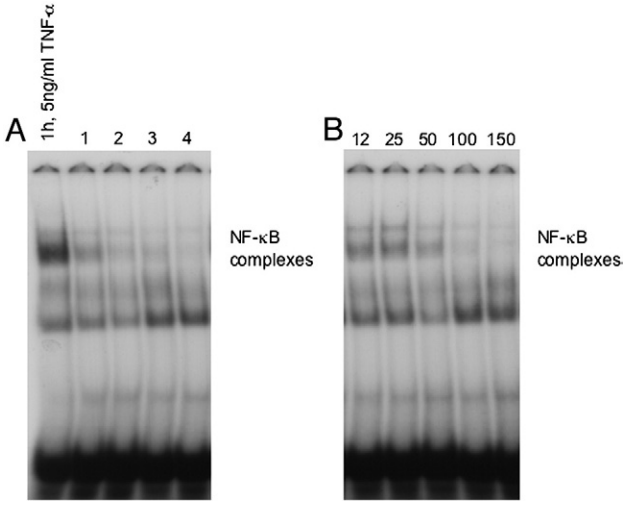

Fig. 3. Time- and dose dependent DMF inhibition of rrTNF-α induced nuclear NF-κB. (A) indicated hours preincubation with 100 μM DMF, followed by rrTNF-α (1 h, 5 ng/ml). (B) 2 h preincubation with indicated μM DMF, followed by rrTNF-α (1 h, 5 ng/ml, $n=3$).

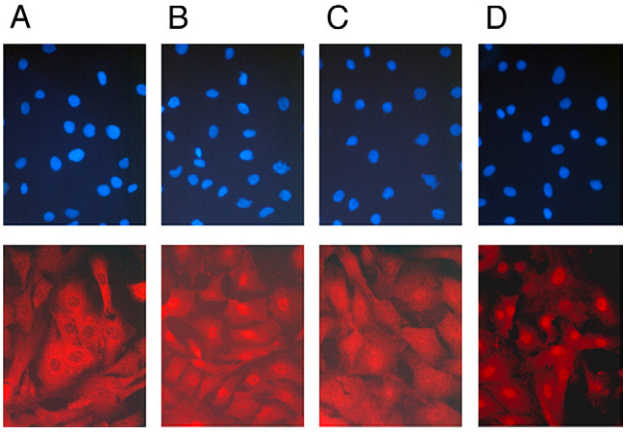

Fig. 5. Immunofluorescence experiments with rat heart endothelial cells: Nuclei stained with Dapi (top line) and for NF-κB (bottom line). In unstimulated control cells (A) most of NF-κB is located in cytoplasm. Stimulation with rrTNF-α 0.1 ng (B) led to a translocation to the nucleus. Pretreatment with DMF (C) could reduce this translocation, whereas pretreatment with MHF (D) decreased the translocation only slightly ($n=5$).

variance (ANOVA) followed by Student–Newman–Keuls test with a 0.05 two-sided significance level. To avoid type II errors (missing significant differences when the sample size is too small) we determined the required sample size for the present study using an approximation for an equal to 0.05 and power to 0.90 (Dawson-Saunders and Trapp, 1990). Accordingly, a sample size of 17 rats in each group had 90% power to detect differences in mean infarct size of 20% (the difference between the mean infarct size in control animals and in DMF-treated animals or in preconditioned animals; infarct size given as percentage of the ischemic risk area) assuming that the common standard deviation is 17% (taken from previous data recorded in identical experimental conditions) (Barbosa et al., 1996) using ANOVA with a 0.05 two-sided significance level. In control experiments in the isolated perfused rat heart we expressed numerical variables as mean±S.D., and compared among groups by ANOVA. Statistical computations were done using Prism software (GraphPad, San Diego, CA, USA; version 3.0a).

3. Results

3.1. EMSA

In cultured rat heart endothelial cells, EMSA experiments revealed that DMF inhibited rrTNF-α and LPS induced NF-κB translocation (in a similar potency like the positive control, PDTC) (Figs. 1–3). Particularly, control experiments with untreated cells showed no translocation of NF-κB (Fig. 1, lane 2). Stimulation with rrTNF-α (lane 3) and LPS (lane 4) provoked a translocation of NF-κB. Pretreatment with DMF or PDTC could block this translocation (lanes 5–8), whereas MHF diminished the translocation only slightly (Fig. 2). Experiments on time dependence demonstrated maximum inhibition after 4 h of preincubation with DMF (Fig. 3A), whereas in experiments on dose dependence a concentration of 100 μM DMF resulted in a complete blocking of the translocation (Fig. 3B). In supershift experiments we could shift the band for NF-κB with the antibody for the p50 subunit completely, whereas the antibodies against the p65 subunit only provoked a shift of part of the band (Fig. 4).

3.2. Immunohistochemistry

In unstimulated RHEC, NF-κB is located in cytoplasm (Fig. 5A), whereas stimulation with rrTNF-α 0.1 ng (Fig. 5B) provoked a translocation to the nucleus. Pretreatment with DMF for 60 min (Fig. 5C) could reduce this translocation to a similar extent as PDTC, which we used as positive control (data not

Table 1
Heart rate *in vivo* (bpm)

	Baseline	Ischemia	Reperfusion
Control	362±27	357±28	346±27
DMF	367±33	366±29	353±28
IPC	378±42	370±38	352±36

Ischemia and reperfusion values are averaged over the entire 45 min resp. 120-minute period.
Values are mean±S.D. of 15, 17 and 17 hearts.

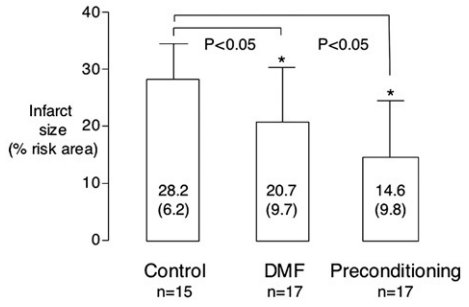

Fig. 6. Myocardial infarct size (% risk area, mean±S.D.). Both, dimethyl fumarate and ischemic preconditioning led to smaller infarct sizes than that of control animals; * = $p<0.05$ vs. control.

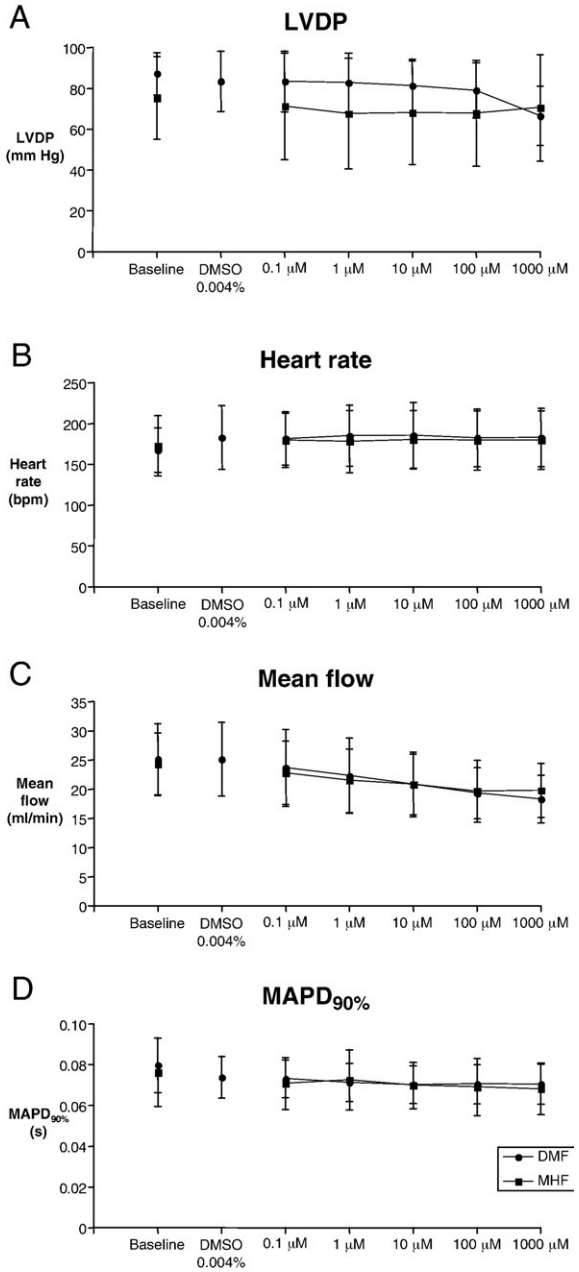

Fig. 7. Dose–response curves of DMF (●) and of MHF (■) in isolated perfused rat hearts. Neither drug significantly affected left ventricular developed pressure (A), heart rate (B), coronary flow (C), or $MAPD_{90\%}$ (D). Values are mean±S.D. of 9 hearts.

shown), while pretreatment with MHF decreased the translocation only to a minor extent (Fig. 5D).

3.3. In vivo-variables

3.3.1. Infarct size

Myocardial infarct size was significantly smaller in rats that had received DMF or ischemic preconditioning than in control rats (independent whether infarct size was expressed as percentage of risk area (20.7±9.7 vs. 14.6±9.8 vs. 28.2±6.2; Fig. 6) or of left ventricular mass (9.1±4.4 vs. 6.7±4.3 vs. 13.0±4.7). Importantly, as required for the infarct data to be valid, the area at risk was similar in all groups.

3.3.2. Electrocardiogram, heart rate, and QT interval

We obtained a standard lead II electrocardiogram in all experiments. The heart rate of the rats before, during, and after ischemia did not reveal any differences among control, DMF-treated and preconditioned rats (Table 1). Similarly, neither QT time (0.064 s±0.012 s; $n=8$ vs. 0.067 s±0.009 s; $n=7$) nor QTc-Bazett (0.1597 s±0.0266 s vs. 0.1719 s±0.0233 s) or QTc-Framingham (0.193 s±0.011 s vs. 0.198 s±0.009 s) differed between DMF-treated and untreated animals.

3.4. Control experiments in the isolated perfused heart

3.4.1. Dose–response curves

In dose–response curves of DMF and MHF, neither drug significantly affected heart rate, coronary flow, left ventricular developed pressure, or $MAPD_{90\%}$ in isolated perfused rat hearts (Fig. 7). Similarly, DMSO 0.004% (the vehicle of DMF) did not affect any of these hemodynamic or electrophysiologic variables.

4. Discussion

Our study demonstrates that DMF inhibits nuclear entry of NF-κB in rat heart endothelial cells and reduces myocardial infarct size following ischemia and reperfusion in rats. This finding indicates a cardioprotective role for DMF, presumably via NF-κB inhibition.

Specifically, infarct size of rats that had received DMF (10 mg/kg body weight as boli i.v. 90 min before ischemia and immediately before ischemia) was significantly smaller compared to vehicle treated control rats. This is the first description of an antinecrotic effect of DMF in rat hearts.

As the endothelium has been proposed to play an important role in ischemia–reperfusion injury, *in vitro* experiments were performed in rat heart endothelial cells. Hypoxia provokes endothelial cells to activate adherence molecules and other proinflammatory factors eventually leading to endothelial cell swelling, platelet activation, microthrombosis and increased vascular tone (Boyle et al., 1996). During reperfusion, those effects may lead to the no-reflow phenomenon, which is characterized by an impaired microcirculation when coronaries are restored (Kloner et al., 1974). A DNA binding study could identify endothelial NF-κB as an essential factor in the regulation of adhesion molecule expression (Collins et al., 1995). Furthermore, a canine myocardial infarction study could demonstrate by immunohistochemistry that NF-κB is primarily localized in vascular endothelium (Sun et al., 2001). Therefore, DMF-induced inhibition of NF-κB in endothelial cells may have limited myocardial infarct size during ischemia/reperfusion in our experiments.

This is the first study demonstrating an effect of DMF on NF-κB activation in cardiac cells. Interestingly only DMF, but not its main metabolite MHF, showed biological activity in our *in vitro* experiments. This corresponds to findings in different cell lines, where also only DMF but not MHF showed an effect on NF-κB activation (Gerdes et al., 2007; Gesser et al., 2007; Loewe et al., 2001).

Activation of NF-κB is thought to play an important role in the pathophysiology of acute myocardial infarction and heart failure (Frantz et al., 2003). Several studies demonstrated beneficial effects of NF-κB inhibition in the setting of myocardial infarction. *In vivo* transfer of NF-κB decoy oligodeoxynucleotides reduced the extent of myocardial infarction following reperfusion in rat and pig hearts (Kupatt et al., 2002; Morishita et al., 1997). In the setting of regional myocardial ischemia followed by reperfusion, gene transfer of IκB limited myocardial infarct size and inflammation in mice (Squadrito et al., 2003) and improved cardiac function in rats (Trescher et al., 2004). In transgenic mice with a blocked activation of NF-κB, myocardial infarct size was significantly reduced compared to wild type mice (Brown et al., 2005).

In experimental models of ischemia/reperfusion injury various pharmacologically active agents decreased the infarcted area, which the authors constitute with NF-κB inhibition through blunting of different NF-κB activating pathways (Izumi et al., 2001; Pye et al., 2003; Squadrito et al., 2003; Thourani et al., 2000). For example, inhibition of the IκB phosphorylation led to a reduction in ischemia/reperfusion injury (Onai et al., 2004). Similarly cyclopentenone prostaglandins inhibited the activation of the IKK complex (Rossi et al., 2000) and reduced myocardial infarct size (Wayman et al., 2002). Furthermore the sesquiterpene lactone parthenolide enhanced the stability of IκB, causing an inhibition of nuclear translocation of NF-κB leading to a reduction of reperfusion-induced myocardial damage in the rat (Zingarelli et al., 2002).

The analysis of heart rate *in vivo* excluded negative chronotropic effects that could have potentially accounted for the cardioprotective effects of DMF in the present study. We could confirm this finding in control experiments in isolated beating rat hearts, in which we demonstrated that DMF and MHF do not exert any acute hemodynamic or electrophysiologic effect that could potentially explain antinecrotic effects of DMF in rat hearts. Specifically, neither DMF nor MHF in concentrations ranging from 0.1 μM to 1000 μM acutely affected heart rate, coronary flow, left ventricular developed pressure, or $MAPD_{90\%}$ in our experiments. Thus, unlike nitrates, beta-adrenergic receptor blockers, or calcium antagonists that increase myocardial blood flow and/or reduced heart rate, DMF appears to diminish myocardial infarct size without acute hemodynamic or electrophysiologic effects.

DMF is an orally available and well-tolerated drug with few severe side effects. Most common side effects are gastro intestinal complaints and flush (Mrowietz and Asadullah, 2005). Particularly no cardiac side effects such as arrhythmias have been described (Mrowietz et al., 1999). This corresponds to our analysis of action potential duration in isolated rat hearts as well as of QTc intervals from the ECG recorded during the *in vivo* experiments in rats. Our results suggest that at least in rats (that is not the ideal species for electrophysiology studies due to species differences in repolarising currents), DMF did not affect ventricular repolarisation and therefore, is unlikely to be pro-arrhythmic. Thus, DMF may offer obvious advantages compared to other approaches to inhibit NF-κB. Gene therapy has still been very controversial (Kupatt et al., 2002; Morishita et al., 1997; Sawa et al., 1997; Squadrito et al., 2003; Trescher et al., 2004). Other pharmacological agents described to reduce infarct size by inhibition of NF-κB activation are not orally available such as heparin (Thourani et al., 2000) or may have serious side effects such as tacrolimus (Squadrito et al., 2000). Therefore, one could postulate beneficial effects of DMF for patients likely to undergo ischemic episodes as during cardiac surgery or due to ischemic heart disease, particularly for acute coronary syndrome.

In the present study, the rat heart served as readily available model to assess acute hemodynamic and electrophysiologic effects. Still, extrapolations from rat to human hearts should be done carefully and absence of electrophysiologic effects should be confirmed in a species that is closer to human beings (e.g. guinea pigs, pigs). Nevertheless, antinecrotic effects of DMF are unlikely to be due to decreased heart rate or left ventricular pressure, increased coronary flow, or shortened action potential duration.

However, the effect of DMF on myocardial infarct size (−7.5%) was not as pronounced as estimated for calculations of sample size. This was presumably related to the fact that infarct size in this study was generally smaller than expected (28% in control rats as opposed to 40–50% in previous studies under similar conditions) (Barbosa et al., 1996). This difference was most likely due to isoflurane that was used as an anesthetic agent in the present study but not in previous experiments. Volatile anesthetics, as isoflurane, have cardioprotective properties and can limit infarct size (Cope et al., 1997). They are able to mimic cardiac preconditioning by priming the activation of mitochondrial K_{ATP} channels via multiple signaling pathways (Zaugg et al., 2002). Still, both DMF and ischemic preconditioning (positive control) reduced myocardial infarct size. This reduction reached statistical significance because not only infarct size but also data variability was lower than in previous studies.

In conclusion, this study demonstrates that DMF inhibits nuclear entry of NF-κB in rat heart endothelial cells and provokes reduction of myocardial infarct size after ischemia and reperfusion in rats *in vivo* that was not due to acute hemodynamic or electrophysiologic effects of DMF. This is the first description of beneficial effects of DMF in rat hearts. Our study suggests that DMF or other drugs modulating NF-κB might be a promising treatment for patients suffering from ischemic heart disease.

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