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European Journal of Pharmacology 435 (2002) 79–83

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Oral bimoclomol elevates heat shock protein 70 and reduces myocardial infarct size in rats

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Received 13 August 2001; received in revised form 12 November 2001; accepted 23 November 2001

Abstract

Bimoclomol has been shown to increase an inducible member of the heat shock protein 70 family (HSP70) and cytoprotect in vitro. Here, we addressed whether oral pretreatment of rats with bimoclomol could elevate myocardial HSP70 and reduce infarct size in a rat model of ischemia and reperfusion. Rats were pretreated with bimoclomol at 3, 6 or 18 h or with 42° thermal stress 24 h before ischemia. Infarct size was significantly decreased 6 h after oral administration of bimoclomol and 24 h after thermal stress. Left ventricles from a separate group of rats were examined for HSP70 levels. Western blots showed a significant increase in HSP70 6 h after oral administration of bimoclomol and 24 h after thermal stress. There was a significant correlation ($P < 0.05$) between HSP70 induction and infarct size reduction, whether produced by thermal stress or oral administration of bimoclomol. Thus, bimoclomol can increase HSP70 and reduce infarct size in a rat model of ischemia and reperfusion.

Keywords: Heat shock protein 70; Ischemia; Reperfusion; Infarct size

1. Introduction

Heat shock proteins are produced by cells in response to a variety of stresses. Thermal stress increases the expression of a number of heat shock proteins which are known to enhance the ability of a cell to survive a subsequent lethal heat challenge (Subjeck et al., 1982; Landry et al., 1982). In addition, thermal pretreatment provides protection from physiological stresses including ischemia and reperfusion. Thermal stress prior to ischemia increases the production of heat shock protein 70 (HSP70) and reduces myocardial infarct size in rat (Donnelly et al., 1992; Hutter et al., 1994) and rabbit (Currie et al., 1993; Marber et al., 1993). Transgenic mice which overexpress rat and human HSP70 are resistant to myocardial ischemia and reperfusion (Marber et al., 1995; Plumier et al., 1995; Hutter et al., 1996).

Pharmacological elevation of stress protein expression could have important therapeutic consequences. Bimoclomol, a novel cytoprotective agent in clinical development for the treatment of diabetic complications, has been shown to increase HSP70 levels and produce cardioprotection in isolated rat hearts (Vigh et al., 1997). However, to date no in vivo study with bimoclomol has demonstrated a direct correlation between myocardial HSP70 induction and a reduction in infarct size.

Thus, the aim of the present study was to examine the effect of bimoclomol and thermal stress on the expression of HSP70 in rat myocardium and correlate these results with myocardial infarct size following ischemia and reperfusion.

2. Materials and methods

All procedures involving animals were conducted following a protocol approved by the Abbott Laboratories Institutional Animal Care and Use Committee and conform to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.1. Experimental protocol

To determine the correlation between cardioprotection and myocardial HSP70, two separate experimental studies were designed. In the first study, five groups of rats received the following treatments prior to ischemia and reperfusion: *Control*, the vehicle (sterile H_2O, 1 ml/kg, p.o.) was dosed

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01552-7

6 h prior to ischemia ($n=10$); *HS42*, rats were subject to thermal stress by raising core temperature to 42 °C for 20 min 24 h prior to ischemia ($n=8$); *Bim3*, bimoclomol (50 mg/kg, p.o.) was dosed 3 h prior to ischemia ($n=6$); *Bim6*, bimoclomol (50 mg/kg, p.o.) dosed 6 h prior to ischemia ($n=6$); *Bim18*, bimoclomol (50 mg/kg, p.o.) dosed 18 h prior to ischemia ($n=7$). In the second study, animals ($n=6$/group) were treated identically to the groups above, except the animals were sacrificed at the end of ischemia and the hearts harvested for quantification of HSP70.

2.2. Determination of myocardial infarct size

Separate groups of control, heat-shocked and bimoclomol treated rats were subjected to 25 min of myocardial ischemia and 3 h of reperfusion. Rats were anesthetized with Inactin (120 mg/kg i.p.). The trachea was intubated and the rats were mechanically ventilated with room air using a small rodent ventilator (Harvard Apparatus, Holliston, MA) at a rate of 70–80 cycles per minute and a tidal volume of 1 ml/100 g body weight. The left femoral artery was cannulated for measurement of mean arterial pressure and heart rate. The left femoral vein was catheterized for drug administration, and the electrocardiogram (ECG) was recorded with the standard Lead II limb leads. Body temperature was maintained at 37 °C using a water circulating heating blanket.

A left thoracotomy was performed, and the heart was exposed through the fifth intercostal space. The pericardium was incised and the heart was gently exteriorized by pressure on the chest, and a 5-0 silk suture was placed around the left main coronary artery near its origin beneath the left atrial appendage. The heart was repositioned in the thoracic cavity with the ligature ends exteriorized and passed through a small length of PE90 tubing to form a snare. For coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery and a bulldog hemostat was applied to the snare for 25 min, resulting in coronary artery occlusion. Myocardial ischemia was confirmed by the presence of ECG changes and by the visual assessment of regional cyanosis and dyskinesis of ischemic region of the left ventricle. Removal of the bulldog hemostat and release of the snare allowed reperfusion of the myocardium, which was maintained for 3 h. Reperfusion was verified by the presence of reperfusion arrhythmias.

At the end of the experiment, the heart was rapidly excised and retrogradely perfused with 3 ml saline to remove any blood. The snare was retightened to reocclude the coronary artery and the heart was retrogradely perfused with a 3 ml solution of 0.5% zinc cadmium sulphide and green fluorescent microspheres (2.26 μm) to mark the ischemic area of the myocardium. The heart was trimmed of the right ventricle and both atria and frozen at −80 °C for 15 min. The heart was transversely sliced into 1–2 mm thick sections from apex to base and incubated in a phosphate buffered 1.5% triphenyltetrazolium chloride (TTC) solution at 37 °C (pH 7.4) for 20 min. The TTC causes viable tissue to stain dark red and the infarcted tissue remains pale. The heart slices were pressed between two Plexiglas plates, removed, and fixed in 10% formalin for 24 h. After fixation, the slices were placed between Plexiglas and both sides of the entire ventricle and the lumen were traced onto acetate sheets. The slices were viewed under long wave ultra-violet light (366 nm) and the fluorescent normal zone was delineated within the slice. Under normal light, the infarct located within the area at risk was traced onto the acetate. The acetates were placed under a microscopic video camera (DEI-750, Optronics Engineering, Goleta, CA) and the resulting images were stored as bitmap files for analysis. The stored images were displayed on a monitor using a Windows-based image analysis software (Image-Pro Plus, Media Cybernetics, Silver Springs, MD), and the areas of the normal zone, area at risk, and the infarct were digitally traced. The area at risk was expressed as a percentage of the left ventricle, and the infarct was expressed as a percentage of the area at risk.

2.3. Determination of myocardial HSP70

Six animals in each experimental group were used for the measurement of HSP70 by Western blot analysis. Control, heat-shocked and rats pretreated with bimoclomol were subject to ischemia for 25 min, at which time the heart was harvested and a small sample of the left ventricle was removed. The samples were minced with a razor blade and transferred to a tissue homogenizer (Tri-R Stir-R, Rockville Centre, NY) with 1 ml of lysis buffer solution (5% sodium dodecyl sulfate (SDS)/1% 2-mercaptoethanol). The samples were homogenized and boiled until the particles were no longer present. The sample was then drawn up into a 1-ml syringe, strained through a 27-gauge needle and quickly frozen in liquid nitrogen.

At the time of analysis, the samples were thawed, and the protein concentration of each sample was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) which was based on a modification of Bradford's dye-binding procedure (Bradford, 1976). Protein samples were diluted 1:1 with a 2 × Laemmli sample buffer solution and 30 μg of total protein from each sample was loaded into lanes of 10–20% linear gradient polyacrylamide gels. Proteins in the samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Equal total protein loads were confirmed by staining untransferred gels with Coomassie blue. Transferred proteins were probed with a primary antibody that was specific for HSP70 (K-20 antibody).

The nitrocellulose was blotted with rabbit anti-goat immunoglobulin G antibody conjugated with horseradish peroxidase. The signal was developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL). HSP70 levels were quantified by densitometry (Personal Densitometer SI; ImageQuant software, Molecular Dynamics, Sunnyvale, CA).

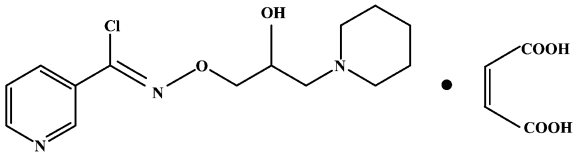

Fig. 1. Chemical structure of bimoclomol-maleate.

2.4. Thermal stress

Rats subjected to thermal stress were placed in a warming box and a rectal thermostat probe was inserted to monitor core body temperature. The body temperature of the rats was increased until a temperature of 41.5–42 °C was reached. This temperature was maintained for 20 min at which time the rats were removed from the warming box.

2.5. Drugs and chemicals

Bimoclomol, (*N*-[2-hydroxy-3-(1-piperidinyl)propoxy]-3-pyridinecarboximidoyl chloride, /*Z*/-2-butenedioate (1:1)) (Fig. 1) was dissolved in sterile H_2O at a concentration of 50 mg/ml. TTC was purchased from Sigma (St. Louis, MO). Green fluorescing polymer microspheres and zinc cadmium sulphide were purchased from Duke Scientific (Palo Alto, CA).

2.6. Statistics

All values are expressed as mean ± S.E.M. Differences between groups in mean arterial pressure, heart rate, rate-pressure product and HSP70 in left ventricle were tested using one-way analysis of variance followed by a post hoc Dunnett's test. $P < 0.05$ was considered significant. Two additional variables were analyzed: infarct size as a percentage of the area at risk and area at risk as a percentage of the left ventricle. For these responses, the control group was compared to the four treatment groups using a one-tailed unpaired *t*-test. A Bonferroni correction was applied to account for the multiplicity of tests. $P < 0.0125$ (0.05/4) was considered significant.

3. Results

3.1. Infarct size and hemodynamic data

Fig. 2A shows the infarct size expressed as a percentage of the area at risk (IS/AAR) in the five treatment groups. The control group had an IS/AAR of 58.9 ± 1.6%. Rats subject to thermal stress of 42 °C for 20 min 24 h prior to occlusion had an IS/AAR of 41.2 ± 7.6% ($P < 0.0125$ vs. control). Pretreatment with bimoclomol (50 mg/kg, p.o.) at 3, 6, or 18 h prior to myocardial ischemia resulted in an IS/AAR of 52.2 ± 3.4% (NS), 45.7 ± 3.7% ($P < 0.0125$ vs. control) and 64.9 ± 5.3% (NS), respectively. Fig. 2B depicts the area at risk as a percentage of the left ventricle. There were no significant differences between the control group and the means of the four treatment groups indicating that similar areas of left ventricular tissue were jeopardized by occlusion of the left coronary artery in each group.

Heart rate, mean arterial pressure, and rate-pressure product for the animals that were part of the infarct size study are shown in Table 1. No significant differences in the baseline

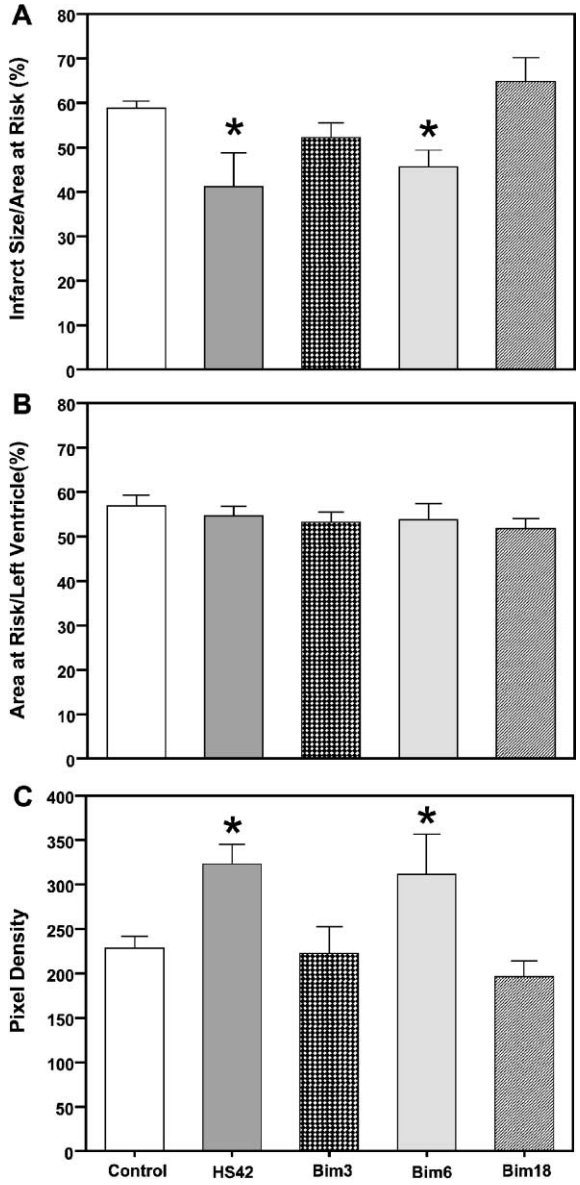

Fig. 2. Each bar represents mean ± S.E.M. of 6–10 animals. *Control*, water (1 ml/kg, p.o.) dosed 6 h prior to ischemia; *HS42*, rats were subject to thermal stress by raising core temperature to 41.5–42 °C for 20 min 24 h prior to ischemia; *Bim3*, bimoclomol (50 mg/kg, p.o.) dosed 3 h prior to ischemia; *Bim6*, bimoclomol (50 mg/kg, p.o.) dosed 6 h prior to ischemia; *Bim18*, bimoclomol (50 mg/kg, p.o.) dosed 18 h prior to ischemia. (A) Infarct size expressed as a percentage of the area at risk. * $P < 0.0125$ from control. (B) Area at risk expressed as a percentage of the left ventricle. (C) Left ventricular HSP70 expressed as pixel density. * $P < 0.05$ from control.

Table 1
Hemodynamic data

	(*n*)	Baseline	Ischemia (min)		Reperfusion (min)			
			Pre-I	20	5	60	120	180
Control	10							
MAP		136 ± 4	129 ± 3	124 ± 4	95 ± 5	90 ± 7	86 ± 5	85 ± 5
HR		399 ± 9	392 ± 8	395 ± 7	374 ± 9	358 ± 12	360 ± 13	347 ± 10
RPP		546 ± 27	507 ± 18	493 ± 22	358 ± 25	329 ± 33	307 ± 20	297 ± 24
HS42	8							
MAP		125 ± 9	120 ± 8	91 ± 11[a]	79 ± 10	86 ± 5	84 ± 4	80 ± 3
HR		409 ± 15	382 ± 24	389 ± 14	366 ± 26	366 ± 16	366 ± 11	353 ± 13
RPP		519 ± 53	465 ± 53	364 ± 53	300 ± 54	321 ± 30	309 ± 22	285 ± 20
Bim3	6							
MAP		139 ± 4	113 ± 8	105 ± 10	92 ± 12	89 ± 8	76 ± 3	85 ± 3
HR		416 ± 9	386 ± 10	394 ± 10	399 ± 8	368 ± 13	377 ± 24	365 ± 23
RPP		576 ± 10	438 ± 36	430 ± 47	368 ± 60	344 ± 41	286 ± 18	250 ± 52
Bim6	6							
MAP		128 ± 7	125 ± 8	115 ± 6	94 ± 11	88 ± 7	86 ± 6	94 ± 8
HR		393 ± 26	395 ± 23	408 ± 16	386 ± 23	390 ± 12	402 ± 11	394 ± 14
RPP		507 ± 51	494 ± 46	466 ± 34	360 ± 61	348 ± 36	344 ± 22	370 ± 34
Bim18	7							
MAP		131 ± 4	128 ± 5	90 ± 12[a]	93 ± 14	95 ± 7	92 ± 8	85 ± 6
HR		437 ± 9	428 ± 11	398 ± 23	383 ± 15	395 ± 12	391 ± 10	393 ± 15
RPP		573 ± 28	552 ± 34	368 ± 72	354 ± 51	380 ± 38	359 ± 35	335 ± 25

(*n*) = number of rats per group; Pre-I = immediately prior to occlusion; MAP = mean arterial pressure (mm Hg); HR = heart rate (beats per minute); RPP = rate pressure product [(mm Hg × beats/min)/100].
Values are mean ± S.E.M.
[a] $P < 0.05$ vs. corresponding time-point in controls.

values of these parameters were observed between groups. When compared to the vehicle control, mean arterial pressure was significantly reduced at 20 min of ischemia in the groups subjected to 42 °C thermal stress and with administration of bimoclomol 18 h before ischemia. The heart rate and rate-pressure products were not significantly different at this time point. The heart rate and mean arterial pressure remained relatively stable throughout the reperfusion period. Mean values were not significantly different among the groups during reperfusion.

3.2. HSP70 data

The data from the Western blots is shown in Fig. 2C. Results are expressed as mean pixel density and show a significant increase in HSP70 24 h after 42 °C thermal stress ($P < 0.05$ vs. control) and a marked increase 6 h after oral administration of 50 mg/kg of bimoclomol. Rats pretreated with 50 mg/kg oral bimoclomol at 3 or 18 h prior to ischemia had similar amounts of myocardial HSP70 as compared with controls.

3.3. Correlation between myocardial HSP70 and infarct size

Fig. 3 is a graph showing the correlation between the amount of left ventricular HSP70 and the infarct size as a percentage of the area at risk of becoming infarcted. There was a linear correlation ($r = 0.93$, $P = 0.02$) between the amount of HSP70 and the observed infarct size reduction.

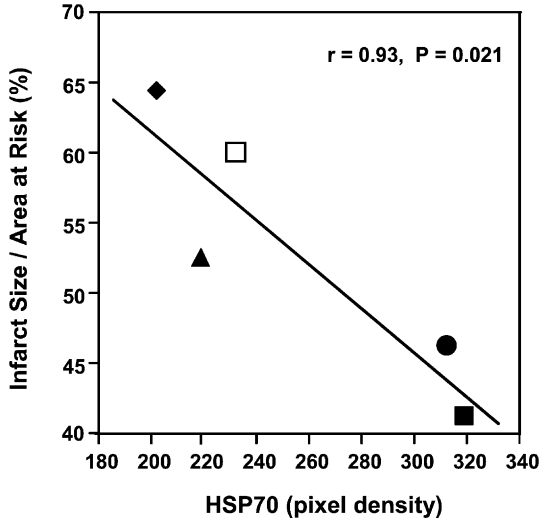

Fig. 3. Correlation between the amount of HSP70 and infarct size expressed as a percentage of the risk area. □, Control; ■, HS42; ▲, Bim3; ●, Bim6; ♦, Bim18.

4. Discussion

A pharmacological approach to elevation of heat shock proteins and subsequent cellular protection could have important therapeutic implications. Bimoclomol, a novel cytoprotective agent, ameliorates the detriment of function in rat models of diabetic neuropathy (Biro et al., 1997) and retinopathy (Biro et al., 1998). Although the mechanism of action for bimoclomol is not clearly defined, in vitro studies have demonstrated a synergistic action of bimoclomol with thermal stress to increase production of heat shock proteins (Vigh et al., 1997). In isolated rat hearts, Vigh et al. (1997) showed a mild induction of HSP70 with ischemia and significant elevations in HSP70 protein levels with ischemia in the presence of bimoclomol. Other investigators have demonstrated in vitro cardioprotection with a structurally related compound (Szabados et al., 2000). In the present study, we demonstrated a significant correlation between HSP70 induction and infarct size reduction, whether produced by heat stress or oral administration of bimoclomol.

Our data demonstrate that there is a window of protection from ischemic injury 6 h after bimoclomol administration but not at 3 or 18 h. If the mechanism of action of bimoclomol were linked to a receptor or ion channel, the compound would have been predicted to be more efficacious at 3 h than at 6 h. Thus, the delay in cardioprotection after oral administration of bimoclomol may be due to the involvement of intracellular signaling pathways leading to the synthesis of heat shock proteins.

As such, the time course for elevation and decline of HSP70 levels with bimoclomol differ from that produced by thermal stress. In the present study, we subjected rats to thermal stress 24 h prior to ischemia and observed a significant induction of HSP70 and reduction in infarct size. The cardioprotective time course was consistent with results from previous studies (Donnelly et al., 1992; Currie et al., 1993; Hutter et al., 1994). However, Qian et al. (1998) demonstrated that rat HSP70 was elevated 4, 12, and 30 h following a 42 °C thermal stress with no cardioprotection observed. In contrast, cardioprotection was observed at 24 h with similar levels of HSP70. The authors argue that cardioprotection cannot be solely explained on the basis of heat shock protein expression but that post-translational modifications, translocation of heat stress proteins, or some other as yet unidentified factor may cause protection.

In conclusion, oral administration of bimoclomol is cytoprotective in a rat model of myocardial ischemia and reperfusion. Further, the compound increases HSP70 in parallel with reduction of infarct size. While a correlation between the reduction in myocardial infarct size and the increase in HSP70 is present, a cause-and-effect relationship does not necessarily exist. However, our results indicate that the cytoprotective actions of bimoclomol are consistent with a long time-course second messenger system, such as HSP70.

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