Original Article

Protection of ischemic myocardium in dogs using intracoronary 2,3-butanedione monoxime (BDM)

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Abstract

Background. – Actomyosin ATPase is one of the major ATP consuming enzymes in the myocardium. We tested whether 2,3-butanedione monoxime (BDM), a reversible inhibitor of actomyosin ATPase, given before coronary occlusion, limits infarct size in anesthetized open-chest dogs.

Methods and results. – After circumflex artery catheterization using fluoroscopic guidance, BDM (125 mM) or buffer vehicle was infused (12.0 ml/min) for 20 min (BDM-20, $n = 5$ and Buffer-20, $n = 6$) or for 5 min (BDM-5, $n = 6$ and Buffer-5, $n = 6$) prior to 60 min of ischemia and 3 h of reperfusion. BDM administration increased subendocardial blood flow 271% above baseline flow (radioactive microspheres), and systolic wall thickening was converted to wall bulging (wall thickening by sonomicrometry was $-27 \pm 29\%$ and $-22 \pm 13\%$ of baseline in BDM-20 and BDM-5, respectively). Adjusted mean infarct size (% area-at-risk) was $11.0 \pm 2.8\%$ and $11.9 \pm 2.6\%$ in BDM-20 and BDM-5 vs. $20.2 \pm 2.5\%$ and $20.5 \pm 2.5\%$ in Buffer-20 and Buffer-5 (ancova, $P < 0.05$ for each BDM vs. Buffer group). Measurement of glycolytic metabolites and the adenine nucleotide pool of myocardium paced electronically at 150 beats per minute during total ischemia at 37 °C following BDM showed a metabolic response similar to that seen in ischemic preconditioning. ATP depletion, nucleoside production, and lactate accumulation were slowed in ischemic tissue treated with BDM.

Conclusion. – BDM given before the onset of ischemia markedly limited infarct size and reduced energy demand after the onset of ischemia. The explanation for the reduced infarct size induced by BDM treatment is hypothesized to be the persistent reduction in energy demand found in ischemic BDM treated myocardium.

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The onset of lethal ischemic injury in myocardium is influenced by the rate of energy metabolism occurring in the ischemic myocardium [1]. Acceleration of metabolism accelerates the transition to irreversibility and vice versa [2]. Thus, the characteristic reduction in energy demand found in ischemic preconditioned myocardium is associated with the delayed onset of lethal injury in this tissue [3].

Most of the major reactions utilizing ATP in myocardium, i.e. the reactions comprising the demand for high-energy phosphates (HEP), have been identified. These include the mitochondrial ATPase, transport ATPases, phosphorylating enzymes such as adenylate cyclase, protein kinase C, etc., and the actomyosin ATPase [3]. Among the known sources of energy demand during myocardial ischemia, the mitochondrial ATPase accounts for about 35–50% [4]. Another potential and variable source of energy consumption early in ischemia is the continued activity of the actomyosin (myofibrillar) ATPase. Continued myofibrillar cross bridge cycling in response to continued electrical stimulation requires energy and is presumed to contribute to the ATP consumption by the ischemic myocyte.

2,3-Butanedione monoxime (BDM) is a reversible inhibitor of actomyosin ATPase. As such, BDM reduces cross-bridge force production. It also reduces myofibrillar sensitivity to calcium and, through these mechanisms, has a negative inotropic effect [5–7]. Nevertheless, systemic administration of BDM is well tolerated. In fact, this agent was introduced

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originally as an antidote for poisoning with organophosphate cholinesterase inhibitors and concentrations up to 30 mg/kg have been used in humans without evidence of systemic or cardiac toxicity [8–11]. It is a small lipophilic molecule (molecular weight = 101.1), which facilitates rapid penetration into the intracellular space.

The studies presented in this paper were done to determine whether intracoronary administration of BDM could protect ischemic myocardium and thereby limit myocardial infarct size in a canine model of ischemia and reperfusion as well as to assess whether the presence of an effective dose of BDM in the myocardium during ischemia reduces energy demand. We report that intracoronary BDM given either 5 or 20 min prior to the onset of ischemia markedly limited myocardial infarct size and that the metabolic response of BDM-treated tissue to total ischemia is similar to that of myocardium preconditioned by ischemia.

1. Methods

All experiments reported here conform to the guidelines of the American Physiological Society regarding the use of laboratory animals and the standards in the "Guide for the Care and Use of Laboratory Animals", DHEW Publ. No. NIH 85-23, revised 1985. Studies were conducted on adult mongrel dogs of either sex, weighing 15–22 kg. All animals used had packed cell volumes greater than 35% and were free of clinically evident disease.

1.1. Surgical preparation

Dogs were pre-medicated with butorphanol tartrate (Torbugesic, Aveco, 0.20 mg/kg i.v.) and then were anesthetized with sodium pentobarbital (approximately 30–40 mg/kg i.v.). After anesthesia, they were intubated and mechanically ventilated using room air supplemented with oxygen. Core body temperatures were maintained at 37–38 °C, by placing dogs on a thermal blanket (#50–7079 Harvard Homeothermic Blanket System, South Natick, MA, USA) controlled by a thermistor probe placed in the rectum. A femoral arterial catheter was placed to measure blood pressure, to obtain blood samples for measurement of arterial blood gases, and to obtain reference samples for measurement of regional myocardial blood flow by microspheres. A femoral venous catheter was placed for administration of normal saline and additional anesthetic as needed. Arterial blood gases were checked periodically and ventilation settings were adjusted as necessary to maintain the blood gases within physiological ranges.

A thoracotomy was performed in the 4th intercostal space and the heart was suspended in a pericardial cradle. The left circumflex coronary artery (LCx) was isolated proximal to its first large marginal branch. An ultrasonic flow probe (#T101 Transonic Systems Inc., Ithaca, NY, USA) was placed on the vessel and a strip of moistened umbilical tape was passed

around the vessel immediately proximal to the flow probe. Occlusion was accomplished by snaring the artery with the umbilical tape into a small plastic tube. Two catheters were inserted into the left atrium for microsphere injection and measurement of atrial pressure. Tween-80 (0.6 ml, 0.05%) was administered to desensitize animals before injection of microsphere suspensions containing this detergent. A micromanometer-tipped catheter (Millar) was inserted into the left ventricle through the apex for measurement of left ventricular pressure and electronic differentiation to yield dP/dt. Mean left atrial pressure, arterial pressure, dP/dt, lead II of the electrocardiogram, circumflex coronary blood flow, and pericardial temperature were monitored throughout the experiment and recorded using a Gould Brush 2400 recorder (Gould, Cleveland, OH, USA).

Two pairs of ultrasonic crystals (Triton Technology, San Diego, CA, USA) were implanted in the region perfused by LCx and the left anterior descending coronary artery (LAD). To measure regional wall thickness, one crystal of each pair was placed in the subendocardium and the other was fixed on the epicardium. The ultrasonic signal was monitored on an oscilloscope (Tektronix Inc., Beaverton, OR, USA) to verify correct crystal alignment. The epicardial crystal was placed directly over the subendocardial crystal, as determined by the minimum signal transit time.

Changes in segment length were recorded at fast chart speed (50 or 100 mm/s). In order to avoid respiration-dependent variability in wall thickness changes, all measurements were made at end-expiration, as determined by simultaneous recording of intratracheal pressure using a pressure transducer connected to the intratracheal tube. Systolic and diastolic wall thicknesses were measured in the last beat before the following inspiration. Wall thickening (WT) was calculated by the formula:

$$\% \ WT = [(EDWT – ESWT)/EDWT] \times 100$$

where EDWT is the end-diastolic wall thickness and ESWT, the end-systolic wall thickness. Using the left ventricular pressure derivative (LV dP/dt) tracing, we determined end-diastolic thickening just before the onset of systole and the end-systolic thickening at maximum negative dP/dt.

1.2. Drug preparation and administration

2,3-butanedione monoxime (BDM) always was prepared fresh immediately prior to infusion. It was dissolved in a HEPES buffer containing in mmoles, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 1.18, EDTA 0.5, Dextrose 12.0, $CaCl_2$ 2.54, and HEPES 200 [12]. After vacuum filtration through a 3 μm millipore filter, the buffer was adjusted to pH = 7.4 using 2N NaOH. Before use, the solution was heated to 38 °C using a 39 °C water bath, and oxygenated for 10 min, bubbling with a gas containing 95% O_2 and 5% CO_2. The temperature during infusion was 37–38 °C.

After a 20-min period of stabilization following surgical preparation, and after intravenous administration of heparin

Table 1
Hearts accepted or excluded from the infarct sizing study[a]

	Buffer-20	BDM-20	Buffer-5	BDM-5
Initial (*n*)	8	11	6	10
Ventricular fibrillation (and resulting death)				
Infusion	0	4 (4)	0	0
Occl.	1 (1)	0	0	1 (1)
Reflow	3 (0)	1 (0)	1 (0)	3 (0)
Other exclusions				
Technical problems	1	2	0	2
Minimal ischemia				1
Final	6	5	6	6

[a] Incidence and timing of ventricular fibrillation, deaths and other exclusions. Ventricular fibrillation occurred during the BDM infusion and before coronary occlusion in four dogs in the BDM-20 group. Ventricular fibrillation occurred either during the coronary occlusion (Occl.) or in the first few minutes of reperfusion (Reflow) in one or more dogs in each group. The numbers in parenthesis are the number of deaths and resultant exclusion resulting from ventricular fibrillation.

(2000 IU), a modified Judkins 6F guiding catheter for right coronary artery catheterization was advanced under fluoroscopic control into the left main coronary artery via a 6-F sheath inserted into the left carotid artery. The Judkins catheter was exchanged with a 3.5F ACS Pinkerton 0.018™ angioplasty catheter over a guide wire selectively inserted into the circumflex artery. The catheter was positioned proximal to the flow meter and coronary occlusion site, while monitoring the electrocardiogram and coronary flow recordings to avoid myocardial ischemia. The balloon of the catheter remained deflated and the lumen was used for saline or drug infusion.

Preliminary experiments were done on six animals to determine a rate of BDM infusion sufficient to abolish myocardial contractility (segmental wall thickening) and to establish the hemodynamic consequences, if any, of this rate of intracoronary infusion. Based on the results of these preliminary studies, a concentration of 125-mM/l BDM and infusion rate of 12 ml/min (1.5 mM/min) was selected. Administration of 3.0 mM/min (*n* = 2) induced complete atrioventricular block or ventricular fibrillation, and lower dosages (0.6 and 1.2 mM/min, *n* = 2 each) failed to consistently inhibit regional contractile function.

1.3. Experiment design: infarction study

Dogs were assigned to either BDM or buffer groups (Table 1). The BDM-treated dogs received 125 mM intracoronary BDM infused at 12 ml/min, to yield a dose of 1.5 mmol BDM/min for either 20 min (BDM-20) or 5 min (BDM-5). Buffer controls received intracoronary buffer at 12 ml/min for either 20 min (Buffer-20) or 5 min (Buffer-5). The circumflex artery was occluded for 60 min immediately after the infusion was completed. The artery then was reperfused for 180 min. The BDM-5 group was studied because four dogs died during drug infusion in the BDM-20 protocol. No deaths or adverse hemodynamic effects occurred during infusion in the BDM-5 group.

1.4. Regional myocardial blood flow

Regional blood flow was measured with 10 ± 1 μm radioactive microspheres at three times in all groups: at baseline (before insertion of the coronary catheter), during the infusions (at 15 min in the 20-min groups and at 2.5 min in the 5-min groups), and 30 min into the 60-min test-occlusion period.

Microspheres (New England Nuclear/Dupont) were agitated using ultrasonic bath for at least 20 min prior use. For each flow measurement, two to three million spheres, labeled with ^{46}Sc, ^{113}Sn or ^{141}Ce, were injected through a left atrial catheter, followed by a 15 ml saline flush. Reference arterial blood samples were withdrawn from the femoral artery at the rate of 7.75 ml/min beginning just before and continuing for 2.5 min after sphere injection.

1.5. Post-mortem studies

At the end of the 3-h reperfusion, heparin (5000 units) and pentobarbital sodium (4 cc) were administered intravenously, and the heart was excised rapidly for ex vivo measurement of area-at-risk, infarct size and regional myocardial blood flow.

Area-at-risk: To determine the anatomical boundaries of the previously ischemic and non-ischemic vascular beds, the circumflex artery was cannulated ex-vivo at the site of occlusion and a second catheter was inserted selectively into the left main coronary artery. Triphenyl tetrazolium chloride (TTC 1%, Sigma) and monastral blue (4%, Sigma) were infused at 37 °C and 120–140 mm Hg in the circumflex and left main coronary arteries respectively. Both TTC and monastral blue were added to a 9.0 mmol/l sodium phosphate buffer at pH 7.4 with 1.0 mmol/l dextran added to maintain physiological intravascular oncotic pressure. The heart then was fixed by coronary perfusion with, and subsequent immersion in, phosphate buffered 3.7% formalin. The fixed hearts were cut into eight transverse slices, which were weighed, and their apical surfaces photographed. The area-at-risk (stained brick red) and area of infarction (non-stained) were identified and traced from an enlarged projection (magnification 8×) of the color slide of each ventricular slice. The area-at-risk and area of necrosis were quantitated using a digitizing tablet interfaced to a personal computer. TTC staining has been shown previously to provide a reliable measure of infarct size in such protocols. However, when TTC was ambiguous, histology was used to verify the correct boundaries.

Regional myocardial blood flow: The ventricular slices were divided into non-ischemic and central ischemic regions for blood flow analysis. Lateral and septal border zones of the area-at-risk were excluded to avoid measurements of flow in tissue having heterogeneous composition. The samples were trimmed of epicardium and divided into inner (subendocardial), middle, and outer (subepicardial) thirds for blood flow measurement. Tissue and reference blood radioactivity were

measured in a gamma counter (Packard A 5912, Downers Grove, IL, USA), with correction for overlap of isotope spectra. Myocardial blood flow was calculated as (tissue counts) X (reference flow)/(reference counts) and expressed as ml/min/gm wet weight.

1.6. Experiment design: metabolic effects of BDM

Our goal in this part of the experiment was to determine if BDM exerted an effect on ischemic metabolism that could explain its cardioprotective effect. To accomplish this aim, we compared the effect of total ischemia at 37 °C in vitro on the rate of anaerobic glycolysis assessed primarily by lactate and glycolytic intermediate accumulation and on the rate of ATP degradation using the method of Jennings et al. [13] in which blocks of myocardium are incubated in a plastic bag immersed in a 37 °C water bath for defined periods of total ischemia. This technique mimics the effect of severe ischemia in vivo and allows the sequential comparison of metabolic changes in both BDM-treated and control tissue of the same heart. In addition, electrical stimulation of the blocks of heart tissue was used to control for the effect of continued electrical stimulation found during ischemia in vivo.

In order to evaluate the metabolic consequences of BDM administration, a separate group of six dogs was given BDM for 20 min using exactly the same technique employed in the infarct-sizing experiment except that ultrasonic crystals were omitted. After 20 min of BDM infusion, the heart was excised quickly (2–3 s) without discontinuing the infusion by cutting across the AV groove with a sharp knife. Within 2–3 s of excision, the heart was plunged into 750 ml of ice cold 0.15 M KCl. Untreated myocardium in the hearts contracted very little in the ice-cold KCl because of the declining temperature and because the bundle of His had been transected. Exactly 60 s after excision, the heart was removed from the cold KCl (heart temperature now 17–23 °C) and the anatomic circumflex and control (anterior descending) bed was excised *en block*. Using a sharp single-edged razor blade, slices of tissue 1–3 mm in thickness were excised quickly from the BDM-treated bed and from the virgin myocardium. These samples were frozen in freon at liquid nitrogen temperature and the time required to freeze the sample with respect to excision of the heart was recorded. Control samples were obtained first and were frozen an average of 45 s after removing the heart from the KCl while the samples of the BDM were frozen 33 s after the control. After excision of the baseline samples, an electrode was placed at the AV groove and on the apical portion of both the circumflex and anterior descending bed. Both beds were placed in separate plastic bags; then, after expelling any air from the bag, each bag was immersed in a 38 °C water bath. The time at which each sample was placed in the water bath was recorded and pacing was begun at a rate of 150 beats/min with a Grass stimulator. Samples of the tissue were taken after exactly 10, 20, 30 and 60 min of ischemia. At each time point, the samples were frozen in freon at liquid nitrogen temperature. All samples were dehydrated while frozen using a Vertis Model 6 Freezemobile Freeze-dryer (Vertis Co. Inc., Gardiner, NY, USA). After drying, the samples were divided into inner and outer halves and myocardial blood flow was determined by the technique described earlier and was reported as milliliters of arterial blood/min/g ww. The dry weight of the samples was converted to wet weight using a water content of 3.68 ml/g dry. In all groups, the tissue samples processed for metabolic assays were from the subendocardial third to half of the myocardial wall. Since the tissue was grossly normal, we used the increased blood flow of the samples treated with BDM to confirm that the samples were from the treated bed. Every sample accepted for analysis had at least twice as much blood flow as the control tissue of the same heart.

The pacing technique was developed after pilot studies in three unpaced hearts failed to show a significant effect of BDM on ischemic metabolism. Baseline results are from six hearts while the effects of ischemia come from the paced hearts.

Metabolite analysis: After shaving the endocardium off of the sample, the subendocardial myocardium was ground into a fine powder in a mortar and pestle. Unpowederable tissue, which consists chiefly of fibrovascular septae, was removed before weighing the powder on a Mettler microbalance. The powder was transferred into a test tube containing 0.6 N perchloric acid. After extracting metabolites, the extract was neutralized with a mixture of KOH and K_2CO_3. The extracts were analyzed for the following metabolites according to enzymatic methods described elsewhere glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), a-glycerol phosphate (a-GP), lactate (L), glucose (G), adenosine triphosphate (ATP), and creatine phosphate (CP) [14,15]. ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HX) and xanthine (X) were measured by high performance liquid chromatography using the technique described by Jennings et al. [13].

All results are reported as μmol/g dry tissue ± the standard error of the mean. Since the dry powder picks up water from the air during weighing, two additional samples of powder were dried to constant weight and the percent water calculated. The weights of the samples used for analysis then were corrected to the true dry weight.

1.7. Statistical analysis

Data are expressed as mean ± SEM. Analysis of variance (ANOVA) was applied to test for possible differences among groups in hemodynamic variables, area-at-risk and collateral blood flow. Differences between means were analyzed with Tukey's test. Repeated hemodynamic measurements within animals were analyzed with anova for repeated measures using Student's *t*-test as a post hoc test when significant differences were shown by anova. To test for differences in the relationship between infarct size and collateral blood

Table 2
Coronary and myocardial blood flow in the infarct sizing study[a]

Group	LCx flow (flow probe) ml/min		Myocardial blood flow (microspheres) inner 2/3; ml/min/g	
	Baseline	Post intervention	Baseline	Post intervention
Buffer-20	26.8 ± 4.4	58.8 ± 4.4^{b}	0.96 ± 0.11	1.26 ± 0.09^{b}
BDM-20	31.6 ± 4.3	82.2 ± 29.1	0.86 ± 0.11	2.27 ± 0.82
Buffer-5	25.7 ± 1.9	44.3 ± 2.4^{b}	1.06 ± 0.06	1.36 ± 0.09^{b}
BDM-5	27.8 ± 3.4	$124.2 \pm 22.7^{b, c}$	0.70 ± 0.07^{c}	$3.35 \pm 0.60^{b, c}$

[a] Effect of intracoronary administration of BDM or buffer on total circumflex (LCx) coronary flow measured by a flowmeter and on myocardial blood flow measured in the inner 2/3 of the circumflex region using microspheres. Blood flow increased significantly during both buffer and BDM infusion but the increase was relatively small in the buffer-treated groups. Note that BDM treatment for either 5 or 20 min resulted in a two- to three-fold increase in flow. The measurements were made after 15 and 2.5 min of infusion respectively, in the 20 and 5-min BDM groups.

[b] $P < 0.01$ vs. baseline.

[c] $P < 0.05$ vs. respective control.

flow, analysis of covariance (ANCOVA) was performed, using infarct size as the dependent variable and collateral blood flow as the independent covariate. Adjusted group means generated by the ancova program were compared using Student's *t*-test. In all analyses, a *P* value equal to or less than 0.05 was considered statistically significant.

2.. Results

2.1. Mortality and exclusions

Of the 35 dogs entered into the infarction study, six died from intractable ventricular fibrillation (Table 1). Four of these six deaths occurred in the BDM-20 group during the infusion of BDM (before the onset of ischemia). Two dogs in other groups developed ventricular fibrillation during coronary occlusion and died. Eight dogs developed ventricular fibrillation at the onset of reperfusion and all eight were defibrillated easily and survived. In addition to the six dogs that died, six other dogs were excluded from data analysis (Table 1). One of these had only minimal ischemia induced by coronary occlusion (subendocardial flow = 0.57 ml/min/gm). Five dogs were excluded because of technical problems that included failure to administer the full dose of the drug (two BDM-20 dogs) and failure to obtain valid measurements of contractile function (two BDM-5 and one Buffer-20 dog). Thus, the results are based on 23 dogs as shown in Table 1.

2.2. Effects of intracoronary BDM or buffer on coronary flow and hemodynamic parameters

Intracoronary infusion of buffer alone was associated with a small increase in coronary flow to the circumflex region, most likely in compensation for the slight hemodilution caused by the buffer (Table 2). In contrast, intracoronary administration of BDM caused a two to three fold increase in coronary blood flow to the circumflex region (Table 2). Neither BDM nor buffer infusion into the circumflex coronary artery (data not shown) affected myocardial blood flow (by microspheres) in the LAD region.

At the end of the drug infusion in the BDM-20 group, blood pressure was decreased and heart rate and left atrial pressure were increased (Table 3). In the BDM-5 group, in contrast, only minor hemodynamic effects were observed at the end of the drug infusion.

2.3. Effect of BDM on myocardial function

BDM infusion abolished regional systolic thickening and caused slight paradoxical systolic thinning before the onset of coronary occlusion in both the BDM-20 and BDM-5 groups (Fig. 1). This functional paralysis was not due to inadvertent ischemia because circumflex coronary blood flow was increased markedly and because no ST changes or cyanosis was noted (Table 2). Administration of buffer at the same infusion rate used with BDM had no deleterious effect on regional contractility, but in contrast, increased wall thickening slightly in the buffer-20 group. Regional contractility in the LAD coronary region (non-treated and non-ischemic bed) was measured in three BDM-20 dogs and in all dogs in the other three groups. It was unaffected by the infusions in the LCx artery (Table 3).

Coronary occlusion was associated with dyskinesis (paradoxical systolic thinning) in all four groups and this severe contractile dysfunction persisted in all four groups throughout the ensuing 3 h of reperfusion. Functional recovery was not enhanced by BDM treatment in either the BDM-20 or BDM-5 groups compared to the respective buffer groups.

2.4. Effect of BDM on baseline predictors of myocardial infarct size

Midway through the 60-min period of coronary occlusion, heart rate continued to be increased in the BDM-20 group vs. the buffer-20 group (Table 3). There were no other significant hemodynamic differences between these two groups. No hemodynamic differences between the BDM-5 group and the buffer-5 group were observed during coronary occlusion. The rate-pressure product (an index of myocardial energy demand) did not differ among any of the groups (ANOVA).

Pericardial temperature during the sustained ischemia recently has been reported to be an important predictor of

Table 3
Hemodynamic data in hearts included in the infarct sizing study[a]

Parameter	Group	Baseline	Post intervention	Mid occlusion	End reperfusion
SBP (mm Hg)					
	Buffer-20	139 ± 8	150 ± 9	120 ± 6	127 ± 6
	BDM-20	133 ± 9	99 ± 14[b]	105 ± 12	117 ± 15
	Buffer-5	158 ± 9	158 ± 11	140 ± 6	142 ± 6
	BDM-5	147 ± 9	124 ± 9	131 ± 4	128 ± 8
DBP (mm Hg)					
	Buffer-20	95 ± 4	97 ± 8	82 ± 4	83 ± 2
	BDM-20	94 ± 5	61 ± 8[b]	85 ± 5	85 ± 12
	Buffer-5	110 ± 6	109 ± 7	100 ± 5	92 ± 7
	BDM-5	99 ± 4	79 ± 5[b]	93 ± 4	90 ± 5
HR (beats/min)					
	Buffer-20	134 ± 9	126 ± 9	132 ± 10	143 ± 7
	BDM-20	140 ± 7	169 ± 8[b]	168 ± 11[b]	169 ± 9
	Buffer-5	152 ± 6	145 ± 5	158 ± 3	167 ± 8
	BDM-5	130 ± 6	152 ± 5	147 ± 6	155 ± 5
LA pressure (mm Hg)					
	Buffer-20	7.2 ± 0.9	9.5 ± 0.8	10.4 ± 1.2	10.8 ± 0.7
	BDM-20	9.8 ± 0.7	16.3 ± 0.9[b]	12.7 ± 0.9	9.1 ± 1.5
	Buffer-5	10.0 ± 0.8	10.5 ± 0.9	11.7 ± 0.7	10.4 ± 0.6
	BDM-5	11.2 ± 1.8	15.2 ± 1.7	14.1 ± 1.7	12.7 ± 2.4
Rate-pressure					
	Buffer-20	188 ± 17	192 ± 19	220 ± 13	184 ± 15
	BDM-20	188 ± 18	170 ± 21	210 ± 14	195 ± 16
	Buffer-5	241 ± 17	228 ± 19	162 ± 13	235 ± 15
	BDM-5	189 ± 17	187 ± 19	191 ± 13	197 ± 15
dP/dt max					
	Buffer-20	1750 ± 139	1975 ± 182	1550 ± 72	1517 ± 120
	BDM-20	1840 ± 206	1660 ± 309	2000 ± 138	1700 ± 63
	Buffer-5	1950 ± 128	1967 ± 143	1817 ± 130	1558 ± 73
	BDM-5	1675 ± 74	1530 ± 99	1516 ± 160	1608 ± 116
Pericardial					
	Buffer-20				37.1 ± 0.3
	BDM-20				37.8 ± 0.6
	Buffer-5				37.2 ± 0.2
	BDM-5				36.9 ± 0.3

[a] Hemodynamic parameters and pericardial temperature in the four groups. Post-intervention values were recorded at the end of the intracoronary infusion. Mid-occlusion measurements were made at 30 min into 60-min ischemic period. End reperfusion = 3 h after reflow. Data are means $\pm$ SEM. SBP and DBP = systolic and diastolic blood pressure, HR = heart rate, LA = left atrial.

[b] $P < 0.05$ vs. respective buffer group.

myocardial infarct size and was not different between groups (data not shown) [16]. Collateral blood flow did not differ significantly among any of the four groups (Fig. 2). The area-at-risk was significantly larger in the BDM-5 vs. BDM-20 group (41 ± 1 vs. $34 \pm 1\%$ left ventricle, $P < 0.05$) but area-at-risk did not differ between both BDM group and its respective buffer control group (Fig. 2).

2.5. Effect of BDM on infarct size

The relationship between infarct size and collateral blood flow for the four groups is shown in Fig. 3. BDM treatment shifted the relationship downward so that both the BDM-5 and BDM-20 groups had smaller infarcts than the buffer-control groups for any level of collateral blood flow ($P < 0.05$ by ancova). Adjusted mean infarct sizes derived from the

ancova are shown in Fig. 4. The groups receiving either 20 or 5 min of buffer infusion had mean infarct sizes of $20.2 \pm 2.5\%$ and $20.5 \pm 2.5\%$ of the area-at-risk, respectively. Infarct size was limited by about 40–50% to $11.0 \pm 2.85\%$ and $11.9 \pm 2.61\%$ of area-at-risk in the BDM-20 and BDM-5 groups ($P < 0.05$ for both BDM groups vs. the respective buffer control group).

2.6. Effect of BDM on myocardial metabolism

Seven hearts were entered into the metabolic study. One was excluded because of ischemia induced in vivo when the catheter was in position in the circumflex artery. The remaining six hearts included in the study were free of ischemia secondary to catheterization and showed the expected increase in coronary flow during perfusion of BDM. Although
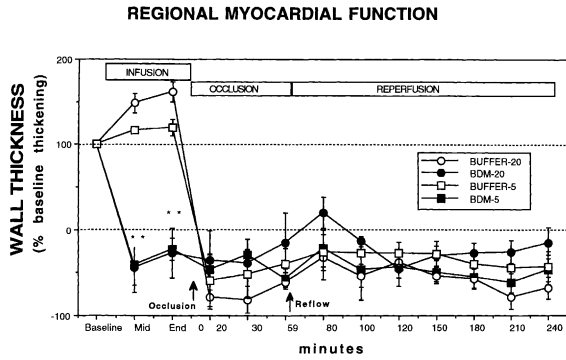
REGIONAL MYOCARDIAL FUNCTION

Fig. 1. Regional myocardial function in the four groups of dogs. Function was evaluated by measurement of wall thickness (sonomicrometry) normalized to the baseline thickening (baseline = 100%). Thickening was measured at 15 (Mid) and 20 min (End) of intracoronary Buffer or BDM administration in the 20-min groups and at 2.5 (Mid) and 5 min (End) in the 5-min groups. Buffer or BDM were administered until the onset of a 60-min circumflex coronary occlusion followed by 180 min of reperfusion. ** $P < 0.01$ for comparisons between the BDM-treated and corresponding buffer-treated groups.

INFARCT SIZE AS A FUNCTION OF COLLATERAL BLOOD FLOW
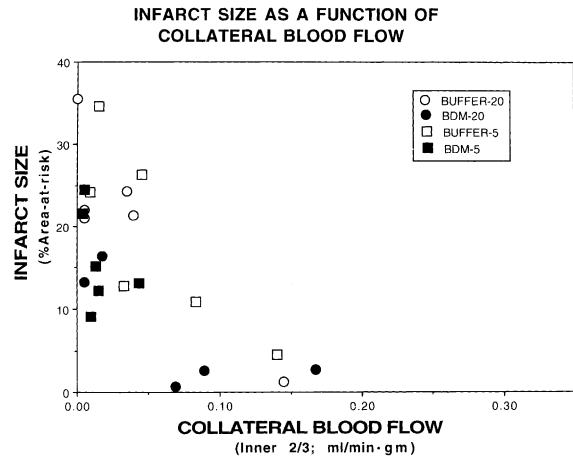
Fig. 3. Infarct size (% of area-at-risk) as a function of collateral blood flow in the buffer-treated (open symbols) and the BDM-treated (solid symbols). The distribution of points is shifted downward in the BDM-treated groups indicating that for any level of collateral blood flow, infarct size was smaller than predicted by the buffer-treated controls ($P < 0.05$ by ANCOVA).

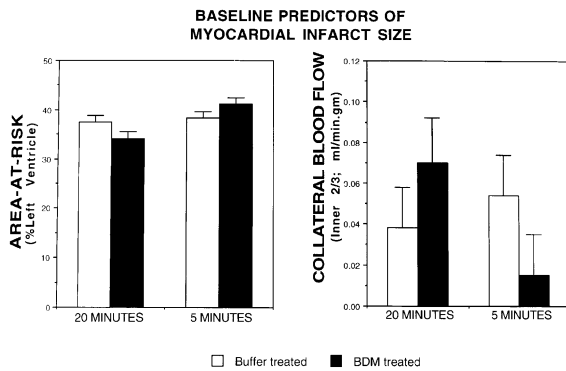BASELINE PREDICTORS OF MYOCARDIAL INFARCT SIZE

Fig. 2. Baseline predictors of myocardial infarct size (collateral blood flow and area-at-risk) in the four groups. The mean area-at-risk was significantly larger in the BDM-5 vs. BDM-20 group ($P < 0.05$ ANOVA), but there was no difference between both BDM-treated group and its respective buffer-treated control group. There was no significant difference in collateral flow among any of the groups.
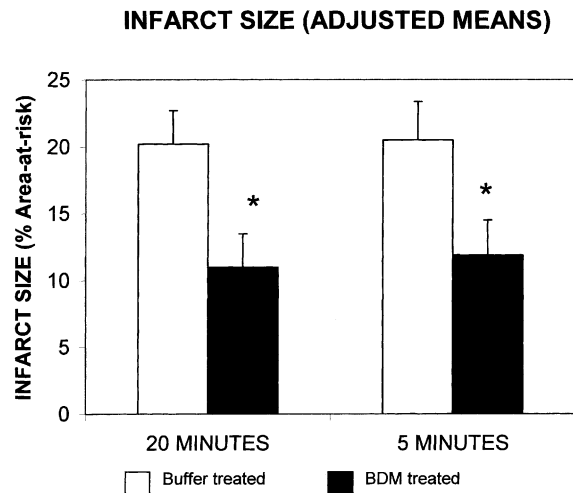
INFARCT SIZE (ADJUSTED MEANS)

Fig. 4. Mean infarct size + SEM in the four experimental groups. The data are the adjusted means derived from ANCOVA of the data shown in Fig. 4. *$P < 0.05$.

sampling was based on anatomical distribution of the coronaries, the fact that an adequate dose of BDM was in the excised tissue was shown by (1) the BDM bed did not contract upon direct mechanical stimulation while the control bed always responded; (2) the BDM perfused bed never contracted in response to electrical stimulation while the virgin myocardium responded by contracting during the first 10–12 min of ischemia; and (3) an increase in coronary flow was present in every piece of BDM treated tissue accepted for analysis (Table 4). The data presented are from the three totally ischemic hearts paced at 150 beats per minute.

Table 4
Measured arterial flow after 15 min of treatment with BDM in samples used for assay of metabolites[a]

	Anterior bed	Posterior bed	P
Control	1.36 ± 0.10	2.45 ± 0.30	0.022
10 min	1.35 ± 0.10	2.85 ± 0.30	0.062
20 min	1.26 ± 0.08	2.62 ± 0.19	0.037
30 min	1.19 ± 0.08	2.57 ± 0.24	0.040

[a] Myocardial regional blood flow (radioactive microspheres) in the anterior left ventricular bed (virgin myocardium) and in the posterior bed (BDM perfused) confirming correct sampling for metabolic analysis. Paired sample *t*-tests show statiscally significant difference ($P < 0.05$) for all samples except for the 10 min sample where the difference is borderline despite a 200% increase in myocardial blood flow.

Table 5
Baseline metabolic data in the BDM treated and control tissue[a]

Metabolites (µmol/g dw)	Baseline (n = 6)	
	Control	BDM
ATP	27.58 ± 0.82	24.66 ± 1.22[c]
CP	14.97 ± 1.51	16.67 ± 1.86
G6P	1.77 ± 0.38	3.01 ± 0.54[b]
Glu	3.59 ± 0.35	10.25 ± 1.40[c]
aGP	3.90 ± 0.58	4.52 ± 0.39
Lactate	16.62 ± 3.78	20.23 ± 2.69
G1P	0.16 ± 0.05	0.20 ± 0.03

[a] These data are from six hearts at baseline including the three hearts that were paced at 150 beats per minute and three hearts that were not paced. The hearts were cooled exactly 60 s in ice-cold isotonic KCl. Then the control and BDM bed were isolated and sampled. The control and the BDM beds were sampled an average of 40 and 80 s, respectively, after removing the heart from the KCl.

[b] The means differ by a paired *t*-test with a probability of P = 0.05 or less.

[c] The means differ by a paired *t*-test with a probability of P = 0.005 or less.

2.7. Effect of BDM administration on baseline metabolites

The effect of ischemia on metabolism was assessed using total ischemia at 37 °C. The heart tissue was paced at 150 beats per minute while ischemic in order to simulate the electrical activity of the heart in vivo and to stimulate a contractile response in any myocardium that was able to contract.

BDM administration did not affect baseline metabolic characteristics except for tissue glucose. This was substantially higher in the BDM treated bed vs. control bed (Table 5). After 20 min of intracoronary BDM infusion, ATP was reduced slightly at baseline compared with the control beds but the difference was not significant. ADP, AMP, ADO, INO, HX, X were similar in control and treated tissue (data not shown). Although not significant, the G6P, G1P, aGP, and lactate of the BDM baseline tissue were generally slightly higher than control. This difference as well as the difference in ATP is attributed to the fact that the BDM-treated tissue was ischemic in a cool state for 31–75 s longer than the control tissue.

2.8. Metabolic effects of ischemia

The rate of ATP depletion and lactate accumulation in the ischemic tissue is shown in Fig. 5. During the first 30 min of ischemia, tissue ATP was lower and tissue lactate was higher in the control tissue than in the BDM treated heart. Also, the rate of adenine nucleotide pool breakdown, as estimated by nucleoside and base production, was lower in the BDM treated tissue (Fig. 6D). As expected from the reduced rate of lactate accumulation, G6P and G1P levels (Fig. 6) rose more slowly in the BDM-treated tissue. aGP was increased markedly by ischemia but the rate of increase was unaffected by BDM. Excess tissue G was consumed during the early phase of total ischemia (data not shown).
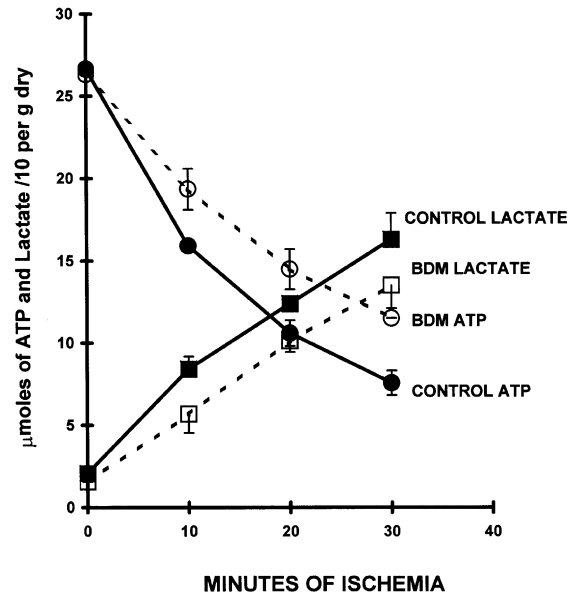
MYOCARDIAL ATP and LACTATE CONTENT DURING 30 MINUTES OF TOTAL ISCHEMIA IN VITRO

Fig. 5. ATP and lactate content of subendocardial samples of myocardium, subjected to total ischemia at 37 °C and paced at 150 beats per minute are shown in this figure. Note that ATP declined more quickly in the control than in the quiescent BDM-treated myocardium. The greatest difference between control and BDM-treated tissue with respect to both ATP depletion and lactate accumulation is noted during the first 10 min of ischemia. Thereafter, the rate of decline in ATP and accumulation of lactate is roughly the same as it is in control tissue ($P < 0.04$ at both 10 and 20 min). Note that the slowed rate of change in control tissue at 20 and 30 min of ischemia roughly coincides with the cessation of the contractile response that was observed after 10–12 min of ischemia had elapsed.

3. Discussion

3.1. Effect of BDM on myocardial infarct size

The results show clearly that intracoronary BDM administration immediately preceding a 60-min coronary artery occlusion effected a striking 40% reduction in myocardial infarct size. Five minutes of BDM infusion was as effective as 20 min of infusion.

Previous studies in large animal models have reported that BDM given during the ischemic period and/or the reperfusion period could limit myocardial stunning or infarction [17–19]. This, however, is the first report that administration of an effective dose of the drug for a short period of time before occluding the coronary artery is protective. Our protocol was designed to allow an optimal test of the effect of BDM. By infusing BDM until the onset of occlusion, we allowed BDM to be trapped in the ischemic myocardium and to exert its beneficial effect throughout the period of ischemia. By stopping the infusion at the onset of occlusion, we avoided the confounding effects of continued slow infu-
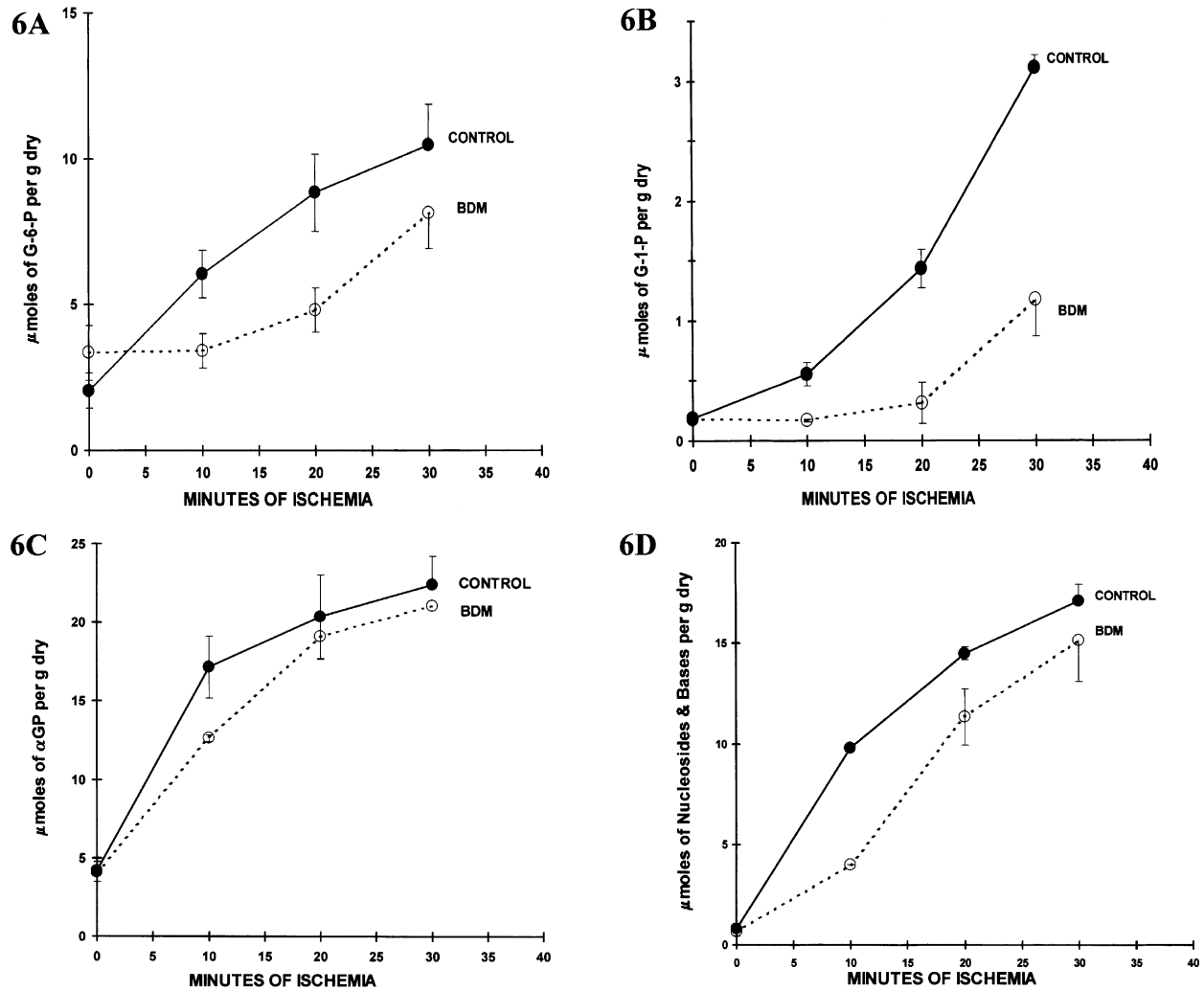
Fig. 6. Glycolytic metabolite content in control and BDM-treated tissue are shown in A–C while nucleoside content is shown in D. The data were obtained from subendocardial samples, all of which had been paced at 150 beats per minute and had been subjected to total ischemia at 37 °C. (A) G-6-P. (B) G-1-P. (C) αGP. (D) Nucleosides and bases. Note that the accumulation of G-6-P and G-1-P was slower in the BDM-treated tissue after 10, 20, and 30 min of total ischemia while aGP accumulated at the same rate in BDM treated and control tissue. The nucleosides and bases (adenosine + inosine + hypoxanthine + xanthine) of the BDM treated tissue accumulated more slowly in the BDM-treated tissue. The greatest difference was noted during the first 10 min of ischemia ($P = 0.002$).

sion during ischemia. Such effects include heterogenous flushing out of ischemic catabolites, which might by itself attenuate ischemic injury. By using selective intracoronary administration we avoided systemic hemodynamic changes and contractile changes in the non-ischemic regions of myocardium. By washing BDM out of the ischemic region by the onset of reperfusion, we have minimized any possible effect of BDM on reperfusion injury.

3.2. Other effects of BDM

In this study, BDM substantially increased coronary blood flow and suppressed regional shortening before the onset of myocardial ischemia. Similar effects have been observed in other studies using large animal models and isolated hearts

[17–21]. Minor hemodynamic effects included slight tachycardia and slight hypotension. Similar effects have been observed in pigs [17].

In this study, despite limitation of infarct size, post-ischemic contractile function was depressed markedly throughout the reperfusion period in BDM-treated as well as buffer-treated hearts. The severe dyskinesis observed in this model is a reflection of a myocardium containing both infarct and viable but stunned myocardium. Limitation of infarct size implies that a greater fraction of the reperfused region is composed of stunned, rather than necrotic myocardium. The persistence of severe dyskinesis in both groups indicates that stunning persisted throughout the reperfusion period in both groups and that BDM did not detectably enhance functional recovery. This is not surprising since several hours to days are required for recovery from severe stunning [22].

3.3. Possible mechanism(s) of beneficial effects of BDM

Energy utilization in myocardial ischemia and its inhibition by BDM: Myocardial contraction is the result of the cyclic interaction between the two contractile proteins actin and myosin. The bridges ("cross-bridges") between the myosin heads and the actin filament repeatedly form and detach, each detachment requiring intervention of the actomyosin ATPase and consumption of ATP. Cross-bridge cycling, responsible for 60 to 80% of the ATP consumption of aerobic myocardium, is not immediately or totally inhibited in ischemia and is thus likely to cause continued ATP consumption in the ischemic tissue [23,24]. This is especially true during the first few minutes of ischemia, i.e. the period during which conduction of the electrical impulse remains intact. Also, the myofibrillar ATPase is activated by norepinephrine through beta adrenergic (cAMP-mediated) and/or alpha adrenergic (protein kinase C-mediated) pathways and both pathways are known to be activated during ischemia [25]. Thus, blockade of the actomyosin ATPase at the onset of occlusion could reduce ATP utilization during ischemia.

2,3-Butanedione-monoxime is known to be a reversible, noncompetitive inhibitor of the actomyosin ATPase in cardiac, skeletal and smooth muscle and thereby inhibits cross bridge cycling [7,26–29]. The degree of inhibition is dose-dependent [26].

Since interventions that reduce energy demand during ischemia such as hypothermia or ischemic preconditioning delay the transition to cell death [30]. Our choice of BDM as a test agent was based on the hypothesis that BDM would delay the onset of ischemic cell death by reducing or eliminating the energy demand generated by cross-bridge cycling. As noted in the first part of this paper, we found that as little as 5 min of BDM infusion reduced infarct size in the canine heart. The next question to be answered was: Did the BDM achieve its beneficial effect by reducing energy demand in the ischemic myocardium i.e., did BDM slow ATP depletion and anaerobic glycolysis during the test period of ischemia? The most direct answer to this question would be obtained by measuring metabolites in myocardium of groups of hearts after 5, 10, 20, and 40 min of severe ischemia in vivo and showing that the BDM treated myocardium responded differently than control myocardium to the episode of ischemia. However, we chose to answer this metabolic question more efficiently by using total ischemia at 37 °C in vitro to mimic in vivo severe ischemia. This technique already has been shown to distinguish the characteristic reduction in energy demand in ischemic canine myocardium that has been preconditioned by ischemia and reperfusion [13]. The advantage of this method is that the effects of multiple intervals of ischemia can be measured and compared to untreated control tissue obtained from the same heart.

When we tested this system in three hearts that were not paced, we did not observe any difference between treated and untreated myocardium. We assumed that this was due to the fact that the myocardium under test was quiescent. Thus, when we made the model more analogous to in vivo severe ischemia by providing electrical stimuli at a rate of 150 beats per minute, we drove the ATPase to consume ATP in the control tissue and observed the slowing in the rate of ATP depletion and anaerobic glycolysis in the BDM tissue presumably because the control tissue was contracting and consuming high energy phosphate at a greater rate than the quiescent BDM-treated tissue.

The results clearly show that the rate of ATP depletion and of anaerobic glycolysis, as assessed by accumulation of lactate, G6P and G1P is slowed when sufficient BDM is present in the tissue to prevent the consumption of HEP involved in contraction.

It is of interest that tissue glucose was higher in the BDM treated tissue than in control. A similar, but greater magnitude increase in baseline glucose, is observed in myocardium preconditioned with 10 min of ischemia and 10 min of reperfusion [30]. A simple calculation reveals that much of this excess glucose is intracellular in the preconditioned heart. However, in the case of BDM treatment, an infusate containing 12 mM glucose is being given at a rate of 12 ml/min to a bed receiving 80–100 ml arterial blood/min at the time the vessel is occluded and the heart is excised. Some of the perfusate glucose as well as the glucose of the blood are trapped in the tissue and its vascular space. The latter space is at least twice that of the untreated control myocardium because of the vasodilation brought about by the effect of BDM on smooth muscle of the arterioles. Thus, with the data available we cannot project if there is an increase in the glucose of the intracellular space. In any event, the increase in intracellular G is less than that seen in preconditioned myocardium. As in preconditioning, much of the tissue G is used during the first 10 min of ischemia, a process that serves to reduce glycogenolysis during the first few minutes of ischemia.

Note that inhibition of the actomyosin ATPase may not be not be the only beneficial effect of BDM on myocardial energy demand, because BDM also has been reported to reduce myofilament sensitivity to calcium and SR calcium release, and to reduce the energetic cost of calcium handling [5,21,31–33].

Substantial evidence for an ATP-sparing effect of BDM during ischemia or metabolic inhibition has been observed previously in isolated perfused or superfused heart tissue. Nayler et al. [34] reported that 30 mM BDM slowed the rate of hypoxia-induced loss of ATP and creatine phosphate in isolated, spontaneously beating rat hearts. Similarly, Hajjar et al. [35] reported that BDM (10 mM) decreased the rate of ATP and creatine phosphate depletion and prevented contracture due to metabolic inhibition in ferret papillary-muscles. Finally, BDM (20 mM) has been shown to reduce the rate of ATP depletion and to prevent ischemic contracture induced by low flow ischemia in isolated perfused rabbit hearts [36].

BDM also has been found to improve cardiac tolerance to cold ischemia in rabbit hearts stored in University of Wiscon-

sin solution [37] or in human cardiac muscle strips incubated in a cold cardioplegic or Krebs–Ringer solutions [24,38]. This beneficial effect is thought to be due to BDM's energy sparing effect.

However, conflicting results also have been published: BDM (5–50 mM) was found to accelerate development of osmotic fragility and to have no effect on the rate of ATP depletion in ischemic, anoxic and/or metabolically inhibited isolated adult rat cardiomyocytes [39,40]. Hebish et al. [41] reported that BDM had a temperature-dependent effect on the energy status of ischemic isolated guinea pig hearts, with protection observed during cold ischemia but absent in hearts at 35 °C.

Possible limitation of reperfusion injury by BDM: In addition to its an energy sparing effect, two studies have provided evidence that BDM may prevent lethal reperfusion injury. Garcia-Dorado et al. [17] reported that BDM administered 3 min before and during 33 min of reperfusion (intracoronary administration of 0.15 to 0.45 mmol/min) in pigs was associated with a 31% limitation of infarct size resulting from a 51 min LAD occlusion and 24 h of reperfusion. Recovery of contractile function also was enhanced. Schlack et al. [19] reported a 73% limitation of infarct size resulting from 60 min of LAD occlusion and 6 h of reperfusion in dogs treated with BDM (intracoronary administration of 2.5 mmol/min) for 65 min starting 5 min prior to reperfusion. The proposed mechanism for efficacy when treatment was confined to the early reperfusion phase was that BDM reduced or prevented the myofibrillar hypercontracture, i.e. it prevented the development of contraction-band necrosis.

Siegmund et al. [40] have reported from studies of isolated rat cardiac myocytes, that hypercontracture induced by 120 min of anoxia and reoxygenation could be prevented by BDM only if BDM was present during the first 15 min of reoxygenation. Treatment of the myocytes with BDM during anoxia, with washout before reoxygenation, was without benefit.

Our study does not permit us to distinguish completely between the energy sparing effect of BDM treatment and prevention of membrane disruption by inhibiting myocyte contracture at the onset of reperfusion.

3.4. Conclusions and implications

We have found that intracoronary 2,3-butanedione monoxime (BDM) given 5 min prior to the onset of ischemia markedly limited myocardial infarct size. This powerful cardioprotective effect is equivalent to the effect of ischemic preconditioning in the same experimental model and most likely occurs through inhibition of the actomyosin ATPase and consequent slowing of energy utilization during the early phase of ischemia. Increased baseline tissue glucose following BDM administration as well as preservation of ATP and slowed anaerobic glycolysis during ischemia clearly mimics the events occurring during in vivo ischemia in myocardium preconditioned by ischemia. Although attenuation of lethal

reperfusion injury by BDM cannot be excluded totally, we do not think that it is a factor because our BDM dose becomes ineffective within 3 s after the infusion is stopped, a fact that suggests that washout of the effective dose of BDM is too fast to prevent reperfusion injury in our model.

The results of this study have possible implications which merit additional investigation. First, since BDM has a rapid onset of action and also can be washed out of the tissue quickly, it is possible that BDM would be of value as a component of surgical cardioplegia in humans undergoing cardiac surgery. Second, since the degree of protection and the metabolic changes observed with BDM are similar to the degree of protection previously observed in this model following ischemic preconditioning, raises the possibility that ischemic preconditioning might be mediated through endogenous inhibition of the actomyosin ATPase [42,43].

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