Protection of ischemic myocardium in diabetics by inhibition of electroneutral Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter

RAVICHANDRAN RAMASAMY,1 JOHN A. PAYNE,2 JOHN WHANG,1 STEVEN R. BERGMANN,1 AND SAUL SCHAEFER3

1Division of Cardiology, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032; and 2Department of Human Physiology and 3Division of Cardiovascular Medicine, Department of Internal Medicine, University of California, Davis, California 95616

Received 27 July 2000; accepted in final form 13 April 2001

Diabetic patients with coronary artery disease have high morbidity and mortality due to cardiovascular complications, including a greater incidence of left ventricular (LV) dysfunction following myocardial infarction (13, 20, 47). Studies using animal models of diabetes, although providing conflicting data on the extent of ischemic injury and infarction in diabetes (10), have consistently shown detrimental alterations in myocardial metabolism and myocyte ion homeostasis (22, 36, 38, 43, 50).

In nondiabetic animals, there are data showing (4, 9, 27, 30, 36, 41, 42, 44, 45) that functional recovery on reperfusion of ischemic myocardium can be enhanced by interventions that maintain tissue ATP availability, and limit derangements in ion homeostasis. The critical role for ion regulation in protecting ischemic myocardium is supported by experiments (2, 14, 28) in which reduced ischemic injury due to global ischemia-reperfusion has been associated with reduced intracellular acidification and lower intracellular sodium and calcium accumulation. Therapeutic interventions that protect ischemic myocardium by limiting ionic derangements have not been completely tested in diabetics, in part due to lack of complete understanding of the alterations in ion transport pathways in diabetics.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
abetic rat hearts by inhibiting the \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransporter with bumetanide and measuring radioactive \( ^{86}\text{Rb} \) uptake, intracellular sodium (by using \( ^{23}\text{Na} \) spectroscopy), intracellular pH, and high-energy phosphates (by using \( ^{31}\text{P} \) NMR spectroscopy) during global ischemia-reperfusion. The data indicate that the flux via \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransporter is increased in diabetic hearts and that inhibition with bumetanide attenuates the rise in intracellular sodium during ischemia and reduces ischemic injury in diabetic hearts.

**MATERIALS AND METHODS**

All experiments were performed with the approval of the Research committee for Animal Care and Use at Columbia University (New York, NY) and the University of California (Davis, CA).

**Rats**

We used spontaneously acute diabetic Bio-Bred (BB/W) rats from a colony maintained at the University of Massachusetts Medical Center (Worcester, MA). The 3- to 4-month-old BB/W rats weighed between 300 and 350 g, with the duration of diabetes being 12 ± 3 days. The rats were receiving daily insulin therapy, which was discontinued 24 h before the isolated heart perfusion studies were performed. The blood glucose levels in these rats were 486 ± 81 mg/dl. The age-matched nondiabetic littermates, also from the colony maintained at the University of Massachusetts Medical Center, were used in this study. The mean blood glucose levels in the littermate controls were 112 ± 12 mg/dl.

**Isolated Perfused Heart Model**

Experiments were performed with the use of an isovolumic isolated rat heart preparation as previously described (34, 36). Acutely diabetic male BB/W rats and nondiabetic age-matched littermates were pretreated with heparin (100 U ip), followed by pentobarbital sodium (65 mg/kg ip). After deep anesthesia was achieved (determined by the absence of a foot reflex), the hearts were rapidly excised and placed into ice-cold saline. The arrested hearts were retrogradely perfused (in a nonrecirculating mode) through the aorta within 2 min. LV developed pressure (LVDP) was determined with the use of a latex balloon in the left ventricle with high-pressure tubing connected to a pressure transducer. Perfusion pressure was monitored with the use of high-pressure tubing off the perfusion line. Hemodynamic measurements were recorded on a four-channel Gould recorder. This design allowed us to rapidly change perfusion media. The hearts were perfused with the use of an accurate roller pump. The perfusate consisted of (in mM) 118 NaCl, 4.7 KCl, 1.2 CaCl\(_2\), 1.2 MgCl\(_2\), and 25 NaHCO\(_3\) (the substrate was 11 mM glucose unless otherwise noted). The perfusion apparatus was tightly temperature controlled, with heated baths used for the perfusate and for the water jacket around the perfusion tubing to maintain heart temperature at 37°C under all conditions. Coronary venous effluent was collected via a cannula that was placed into the pulmonary artery. PO\(_2\) was measured in the effluent with the use of a pH-blood gas analyzer (model IL 1306, Instrumentation Laboratories). Myocardial oxygen consumption was calculated as (0.003 × arterial PO\(_2\) − 0.003 × effluent PO\(_2\)) × total flow/LV weight.

**Choice of Bumetanide to Inhibit Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter**

Several loop diuretics (such as bumetanide, furosemide, piretanide, and benzmetanide) are available to inhibit Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter. Among the diuretics, bumetanide has been shown to be specific and well characterized for its ability to inhibit the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter from several organs and cells [see the recent review by Russell (40)]. At concentrations of 10\(^{-6}\)–10\(^{-7}\) M, bumetanide (inhibitory constant \( K_i \approx 1 \times 10^{-6} \) M) has been shown to inhibit Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter in several organs, including the heart (19, 40). In this study, we used 5 \( \mu \)M bumetanide to inhibit the myocardial Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter.

**Measurements of \(^{86}\text{Rb} \) Uptake via Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter**

Bumetanide-sensitive \(^{86}\text{Rb} \) (an analog of K\(^+\) ) uptake was measured by perfusing hearts with radioactive \(^{86}\text{Rb} \) (0.1 mCi/ml) and cold RbCl (1 mM) under recirculating conditions for 1 h. Hearts from diabetic \(( n = 6 )\) and nondiabetic rats were perfused with \(^{86}\text{Rb} \) buffer for 60 min to measure the uptake of \(^{86}\text{Rb} \) by all K\(^+\) pathways. To measure the \(^{86}\text{Rb} \) uptake via the bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, diabetic \(( n = 6 )\) and nondiabetic \(( n = 6 )\) hearts were perfused with the buffer containing \(^{86}\text{Rb} \) and bumetanide for 60 min. The uptake of \(^{86}\text{Rb} \) in the absence and presence of bumetanide was measured in the hearts at the end of the perfusion period in a gamma-well counter. Hearts were perfused with the cold Krebs buffer (without any RbCl) for 2 min before being counted to remove \(^{86}\text{Rb} \) from the vasculature. Myocardial \(^{86}\text{Rb} \) uptake was quantified by normalizing for perfusate radioactivity and heart weight.

**Na\(^+\)-K\(^+\)-2Cl\(^-\) Protein**

Hearts \(( n = 6 \) in each group\) were homogenized in the presence of protease inhibitors, and membranes were prepared by differential centrifugation as previously described (49). Membranes were analyzed by Western blot after separating samples on 7.5% Tricine gels and electrophoretic transfer to polyvinylidene difluoride membrane (Immobilon-P, Millipore). Equal amounts of protein (100 mg of protein) were loaded from each sample (diabetic or nondiabetic hearts). The anti-Na-K-Cl cotransporter monoclonal antibody (T4 antibody) (23) and enhanced chemiluminescence assay (ECL, Amersham) was used to detect the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter from several preparations. Western blots were compared among the groups to get a qualitative estimate of changes in protein expression.

**Creatine Kinase**

Creatine kinase (CK) was measured from timed 5-min collections of the effluents for 60 min of reperfusion after the ischemic period. Each 5-min collection was analyzed with the use of established spectrophotometric methods (34, 41). Total integrated CK activity over the reperfusion was calculated for each heart and corrected for the dry weight of the heart. CK release was expressed in units per gram of dry weight. Total CK activity was calculated by measuring tissue CK activity and integrated CK release on reperfusion. The percentage of CK released on reperfusion was calculated as %CK release on reperfusion

\[ = \frac{\text{CK release on reperfusion}}{\text{total CK activity}} \times 100 \]

This normalization of CK release enabled us to correct for lower total myocardial CK content in the diabetic hearts.

**NMR Spectroscopy**

\(^{31}\text{P} \) NMR spectroscopy. All spectroscopy were performed on an AMX 400 (Bruker) or Omega-300 (General Electric) ver-
Statistical Methods

Data were analyzed with the use of INSTAT software (GraphPad; San Diego, CA) operating on an IBM-compatible personal computer. The differences among different groups were assessed with the use of analysis of variance (ANOVA) for repeated measures, with subsequent Student-Newman-Keuls multiple comparisons post tests if the P value for ANOVA was significant. P < 0.05 was used to reject the null hypothesis. All data are expressed as means ± SD.

Results

Cation Flux Via Na⁺-K⁺-2Cl⁻ Cotransporter in Diabetic Hearts

86Rb uptake studies. Because Rb⁺ is an analog of K⁺, measurements of radiotracer Rb⁺ uptake was undertaken to estimate the flux via the Na⁺-K⁺-2Cl⁻ cotransporter in perfused hearts. Figure 1 illustrates the baseline measurements of 86Rb uptake in diabetic and nondiabetic hearts. In diabetic hearts, bumetanide reduced the uptake of 86Rb by ~50%, consistent with an increase in flux via the Na⁺-K⁺-2Cl⁻ cotransporter in diabetic hearts. In comparison, bumetanide did not significantly reduce 86Rb uptake in nondiabetic hearts.

Western blot analysis of the Na⁺-K⁺-2Cl⁻ cotransporter levels. Because one potential explanation for the greater inhibition of Na⁺-K⁺-2Cl⁻ cotransporter flux in diabetic hearts is greater levels of the protein, we measured the cotransporter by using Western blot techniques with a polyclonal antibody specific for the Na⁺-K⁺-2Cl⁻ cotransporter. Western blot analysis using a mouse monoclonal anti-Na⁺-K⁺-2Cl⁻ cotransporter antibody (T4) was performed on membranes prepared from control and diabetic hearts (Fig. 2). Similar to the observations by Lytle et al. (23) on other
cell types, two major bands were observed prominently in the control hearts. These two bands represent mature glycosylated protein present at the plasma membrane (upper band; ~170 kDa) and immature unprocessed protein (lower band; ~135 kDa). The intensity of the mature protein, in arbitrary units, was 176 ± 36 in diabetic and 162 ± 55 in control hearts. These data demonstrate no significant differences in the mature Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter expression in the diabetics and controls. Thus the elevated Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter activity observed in the diabetic hearts cannot be explained by an increased level of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter protein expression.

Impact of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) Cotransporter Inhibition on Cardiac Function and Ischemic Injury

Functional changes during ischemia-reperfusion. LVDP and EDP were similar in all groups under baseline conditions (Table 1). Ischemia resulted in significant reductions in LVDP in all groups. The rise in EDP during ischemia was attenuated in the bumetanide-treated diabetics compared with other groups of hearts. During reperfusion, the bumetanide-treated diabetic hearts exhibited greater LVDP recovery than the untreated diabetic, nondiabetic control, and bumetanide-treated nondiabetic hearts. Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter inhibition did not affect myocardial oxygen consumption.

Table 1. Hemodynamic values from isolated rat hearts

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Baseline</th>
<th>Ischemia, 20 min</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>10</td>
<td>108 ± 17</td>
<td>60 ± 12‡</td>
<td>21 ± 15‡</td>
</tr>
<tr>
<td>LVDP</td>
<td></td>
<td>11 ± 3</td>
<td>34 ± 6‡</td>
<td></td>
</tr>
<tr>
<td>EDP</td>
<td></td>
<td>27.6 ± 2.9</td>
<td>22.9 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>MVO(_2)</td>
<td></td>
<td>122 ± 21</td>
<td>38 ± 9‡</td>
<td>76 ± 12‡‡</td>
</tr>
<tr>
<td>DM + BUM</td>
<td>9</td>
<td>9 ± 2</td>
<td>14 ± 6†</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>LVDP</td>
<td></td>
<td>25.9 ± 4.5</td>
<td>26.9 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>EDP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVO(_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondiabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>12</td>
<td>115 ± 19</td>
<td>56 ± 14‡</td>
<td>16 ± 19‡</td>
</tr>
<tr>
<td>LVDP</td>
<td></td>
<td>11 ± 4</td>
<td>74 ± 19‡</td>
<td></td>
</tr>
<tr>
<td>EDP</td>
<td></td>
<td>29.2 ± 6.7</td>
<td>26.9 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>MVO(_2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON + BUM</td>
<td>11</td>
<td>111 ± 24</td>
<td>57 ± 21‡</td>
<td>29 ± 23‡</td>
</tr>
<tr>
<td>LVDP</td>
<td></td>
<td>11 ± 4</td>
<td>26.7 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>EDP</td>
<td></td>
<td>66 ± 18‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVO(_2)</td>
<td></td>
<td>31.9 ± 6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of rat hearts in each group. LVDP, left ventricular developed pressure; EDP, end-diastolic pressure (expressed in cmH\(_2\)O pressure); MVO\(_2\), myocardial oxygen consumption (expressed as mmol·g dry wt \(^{-1}\)·min \(^{-1}\)); DM, diabetic; BUM, bumetanide; and CON, control. \(^*\)P < 0.05, LVDP recovery during reperfusion was significantly greater in DM-BUM hearts vs. DM, CON, and CON + BUM hearts. \(^‡\)P < 0.05, EDP was significantly lower in DM-BUM hearts vs. DM, CON, and CON + BUM hearts. \(^\dagger\)P < 0.05, significantly different from their respective baseline values.

Table 2. Creatine kinase data

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Total CK Activity</th>
<th>CK Release On Reperfusion</th>
<th>%CK Release On Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>10</td>
<td>798 ± 199</td>
<td>502 ± 102</td>
<td>62.9 ± 5.2</td>
</tr>
<tr>
<td>DM-BUM</td>
<td>9</td>
<td>756 ± 216</td>
<td>156 ± 36</td>
<td>24.6 ± 4.5†</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>12</td>
<td>1,583 ± 451†</td>
<td>892 ± 227</td>
<td>56.3 ± 6.7</td>
</tr>
<tr>
<td>CON-BUM</td>
<td>11</td>
<td>1,396 ± 292†</td>
<td>828 ± 141</td>
<td>59.3 ± 8.1</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of rat hearts in each group. CK, creatine kinase. CK activity is expressed as IU/g dry weight. See MATERIALS AND METHODS for measurements of total myocardial CK activity and for estimation of %CK release on reperfusion. \(^*\)P < 0.05 vs. all other groups for %CK release; \(^\dagger\)P < 0.05 vs. DM and DM-BUM for total CK activity.

Ischemia-Reperfusion Injury

CK release, a measure of ischemia-reperfusion injury, was measured during reperfusion in all the groups of hearts studied. Consistent with previous data, diabetic hearts had a lower CK release than nondiabetic control hearts (Table 2). Since it was earlier demonstrated (26, 33) that the total amount of myocardial CK and its activity is lower in streptozotocin-induced diabetic animals, we measured the total myocardial CK activity in these hearts and normalized the release of CK during reperfusion. As demonstrated (26, 33) with the use of chemically induced diabetic animal models, we also show that the total myocardial CK activity is significantly lower in the genetically diabetic BB/W rat hearts. However, when expressed as a percentage of total CK activity, it can be seen that the both diabetic and nondiabetic hearts released ~60% of total CK on reperfusion (Table 2). Bumetanide markedly reduced the fraction of CK released in the diabetic hearts but did not limit CK release in the nondiabetic hearts. These data demonstrate that inhibition of the bumetanide-sensitive Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter protected the diabetic hearts from ischemia-reperfusion injury.

Contribution of Flux via Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) Cotransporter Toward Changes in Intracellular Sodium During Ischemia-Reperfusion

\(^{23}\)Na NMR studies were conducted to investigate the contribution of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter pathway to the changes in intracellular sodium during ischemia in diabetic and nondiabetic hearts. Relative changes in intracellular sodium in each group of hearts during baseline, ischemia, and reperfusion are displayed in Fig. 3. In each group, baseline intracellular sodium was set to 100%, and the changes in sodium during ischemia-reperfusion are reported relative to the baseline values. The intracellular sodium increased to 229.8 ± 18.1% in diabetic hearts compared with a greater increase of 309.1 ± 14.2% in nondiabetic hearts (P < 0.05).

Inhibition of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter with bumetanide significantly attenuated the rise in intracellular sodium during ischemia in both diabetic and

AJP-Heart Circ Physiol • VOL 281 • AUGUST 2001 • www.ajpheart.org
nondiabetic hearts, with the end-ischemic [Na] being 130.2 ± 12.2% in diabetes-bumetanide and 258.2 ± 13.5% in the control-bumetanide hearts. Comparing changes in intracellular sodium in the presence and in the absence of bumetanide, it is evident that the Na⁺-K⁺-2Cl⁻ cotransporter contributed ~18% of the rise in sodium during ischemia in control hearts, whereas the Na⁺-K⁺-2Cl⁻ cotransporter was responsible for ~44% of the increase in sodium during ischemia in diabetics. These results clearly indicate that the flux via Na⁺-K⁺-2Cl⁻ cotransporter was increased in diabetic hearts.

**Metabolic Consequences of Na⁺-K⁺-2Cl⁻ Cotransporter Inhibition**

**Phosphocreatine.** All hearts had rapid loss of phosphocreatine (PCr) at the onset of global ischemia and partial return of PCr levels on reperfusion. Treatment with bumetanide increased the levels of PCr more in diabetic than in nondiabetic hearts (Fig. 4). Bumetanide did not affect the loss or return of PCr in nondiabetic hearts.

**ATP.** ATP, expressed as a fraction of baseline (Fig. 5), was similar in diabetic and bumetanide perfused diabetic hearts before ischemia. During ischemia, ATP content was preserved in bumetanide-treated diabetic hearts, whereas in all other groups, ATP loss was quite severe. At the midpoint of the reperfusion period, bumetanide-perfused diabetic hearts had significantly higher levels of ATP than untreated diabetic hearts (fraction of baseline ATP was 0.52 ± 0.07 in diabetes-bumetanide vs. 0.16 ± 0.04 in diabetic hearts, *P* < 0.05). In nondiabetic hearts, inhibition of the Na⁺-K⁺-2Cl⁻ cotransporter with bumetanide did not conserve ATP levels during ischemia or reperfusion.

**Intracellular pH during ischemia-reperfusion.** Bumetanide perfusion did not alter intracellular pH in diabetic hearts before ischemia (Table 3). However, during ischemia, bumetanide-perfused diabetic hearts became less acidotic than untreated diabetic hearts, resulting in an end-ischemic pH of 6.02 ± 0.03 in diabetes versus 6.38 ± 0.02 in diabetes-bumetanide hearts (*P* < 0.05). Reperfusion resulted in pH recovery to baseline values in both hearts within 10 min.

Intracellular pH was not affected by bumetanide perfusion under any condition in nondiabetic hearts. The end-ischemic pH was 5.98 ± 0.02 in control versus Fig. 3. Changes in intracellular sodium concentration ([Na]i), expressed as a percentage of baseline during 20 min of global ischemia and 40 min of reperfusion in diabetic (A) and nondiabetic (B) heart groups. In the untreated diabetic and nondiabetic group, 9 hearts were studied, whereas in the bumetanide-treated group 6 hearts were studied. Each arrow represents the data obtained every 5 min. *P < 0.05, significantly greater than the bumetanide-treated group. Baseline intracellular sodium was significantly lower than those obtained for the entire ischemia period and during the first 20 min of reperfusion within the respective groups.

Fig. 4. Phosphocreatine (PCr), expressed as fraction of baseline, in DM, CON, DM-BUM, and CON-BUM hearts during baseline and at the end of reperfusion. These data were obtained from 31P nuclear magnetic resonance (NMR) measurements. At the end of ischemia, all groups of hearts had no NMR-measurable PCr levels. *P < 0.05, bumetanide-treated diabetic hearts had significantly higher levels of PCr than other groups. Baseline levels of PCr measured with the use of HPLC and, expressed in micromoles per gram dry weight, were 34.89 ± 5.07 in CON and 34.55 ± 4.66 in DM hearts (n = 6 in each group). #, @, ∅, *P < 0.05 vs. baseline values in its group.
weight, was significantly lower in DM compared with CON hearts measured by HPLC and, expressed as micromoles per gram dry baseline values in its group. Baseline concentration of ATP was group).

Increased Na\(^+\)-K\(^+\)-ATPase activity in the presence of active sodium influx via other transporters results in an increased baseline [Na\(_i\)]. In the current experiments, the baseline \(^{86}\)RbCl uptake measurements demonstrated an increased flux via the bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter in diabetic hearts, findings that mirror a report (25) that demonstrated a 70% increase in flux via the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter in aortas from diabetic rats. The flux studies using \(^{86}\)Rb\(^+\) (K\(^+\) analog) and bumetanide indicate that the net flux via the bidirectional Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter is directed toward increasing intracellular ion concentration (Na, K, and Cl).

An increase in the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter activity could be due to an increase in the levels of the protein or to activation by endogenous factors, such as changes in cell volume, and cAMP-dependent, and non-cAMP-dependent protein phosphorylation (3, 6, 29, 31). The Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter protein levels measured by western blots were similar in diabetic and nondiabetic hearts, indicating that the observed increases in the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter activity were likely due to activation by endogenous factors.

Increased Flux Via Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter and Intracellular Sodium During Ischemia

The changes in intracellular sodium during ischemia result from the balance of changes in Na\(^+\) influx and efflux. Figure 3 shows that bumetanide decreased peak intracellular sodium in diabetic and nondiabetic hearts. The magnitude of the decrease in intracellular sodium, due to bumetanide treatment, is greater in diabetics than in nondiabetics. These findings are consistent with a greater contribution of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter to the increase in intracellular sodium during ischemia in diabetic hearts. Two studies support our observations that the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter may play a greater role in the influx of sodium during ischemia when Na\(^+\)-K\(^+\)-ATPase activity is reduced. Anderson et al. (1) have shown that inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter with bumetanide decreased the intracellular Na\(^+\) uptake and accumulation during ischemia in ouabain-treated neonatal hearts. Similarly, Rubin and Navon (39) demonstrated

<table>
<thead>
<tr>
<th>Hearts</th>
<th>Baseline</th>
<th>Intraacellular pH at End of Ischemia</th>
<th>Reperfusion 5 min</th>
<th>Reperfusion 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>7.01 ± 0.04</td>
<td>6.04 ± 0.48‡</td>
<td>6.79 ± 0.06‡</td>
<td>7.05 ± 0.07</td>
</tr>
<tr>
<td>DM-BUM</td>
<td>7.04 ± 0.09</td>
<td>6.24 ± 0.04‡§§</td>
<td>6.77 ± 0.09†</td>
<td>7.02 ± 0.06</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>7.03 ± 0.02</td>
<td>5.95 ± 0.06§</td>
<td>7.02 ± 0.08</td>
<td>7.04 ± 0.09</td>
</tr>
<tr>
<td>CON-BUM</td>
<td>7.01 ± 0.06</td>
<td>6.01 ± 0.08§§</td>
<td>6.98 ± 0.06</td>
<td>7.06 ± 0.06</td>
</tr>
</tbody>
</table>

Data are means ± SE. Six rat hearts were used per group. *P < 0.05 vs. CON, CON-BUM, and DM for end of ischemia data. †P < 0.05 vs. CON and CON-BUM for the 5-min reperfusion period. ‡P < 0.05 vs. their respective baseline values. §P < 0.05 vs. 5-min time period of reperfusion in its group.

In summary, the results of the present study show that the flux via Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter is higher in hearts isolated from diabetic animals under baseline and ischemic conditions. As a consequence, inhibition of the cotransporter with bumetanide limits the rise in intracellular sodium, conserves ATP and limits acidosis during ischemia, and reduces ischemic injury. The mechanisms of these responses can be postulated in light of known effectors of sodium regulation in the heart.

**Increased Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter Flux and Intracellular Sodium in Diabetic Hearts**

Steady-state [Na\(_i\)] is maintained by a balance between sodium influx and efflux. Under baseline conditions, sodium influx occurs via the sodium channels, the Na\(^+\)-H\(^+\) exchanger, the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, and the Na\(^+\)/Ca\(^{2+}\) exchanger, whereas sodium efflux occurs mainly through the Na\(^+\)-K\(^+\)-ATPase (19). In hearts from diabetic animals, inhibi-
that the rise in intracellular sodium, due either to inhibition of Na\(^+\)-K\(^+\) ATPase or to hypothermic ischemia, could be attenuated by treatment with furosemide (an inhibitor of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter). That study also showed that inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter protected the hearts during hypothermic preservation (39). These studies support our findings that in diabetic hearts with reduced Na\(^+\)-K\(^+\)-ATPase activity, inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter reduces the rise in intracellular sodium during ischemia.

**Inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter on Acidosis, ATP, and Ischemic Injury**

In this study, bumetanide perfusion resulted in both attenuation of intracellular sodium and acidosis, as well as higher levels of ATP, in diabetic hearts during ischemia. Potential reasons for reduced acidosis during ischemia include reduced proton production from anaerobic glycolysis, reduced ATP hydrolysis, or greater proton efflux from ion transport mechanisms (7, 11, 48). While it is difficult to elucidate the precise sequence of events that resulted in less acidosis, greater ATP preservation, and a lower rise in intracellular sodium during ischemia, it is possible that the requirements of ATP for transport pathways to maintain intracellular sodium homeostasis may have been attenuated in bumetanide-treated diabetic hearts, thus impacting net changes in acidosis and ATP levels. One possible scenario is that the reduction in overall intracellular sodium load during ischemia, as well as during reperfusion, may facilitate proton exchange and alter sodium gradient thereby reducing intracellular calcium load. Attenuation of intracellular calcium accumulation has been associated with greater ATP production by the mitochondria (45).

Inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter with bumetanide reduced ischemia-reperfusion injury by \~50% in diabetic hearts, while the reduction was \~20% in nondiabetic hearts treated with bumetanide. These data suggest that inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter with the use of bumetanide was protective only in diabetic hearts. The likely explanation can be postulated in light of higher activity of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter and its contribution to Na\(^+\) uptake in diabetic hearts. The data presented here is consistent with earlier studies (2, 21, 35, 45), which demonstrated protection of ischemic myocardium by interventions that reduce intracellular acidification, and lower intracellular sodium and calcium accumulation.

**Limitations**

The findings from our isolated perfused heart experiments, demonstrating protection of ischemic myocardium in diabetic by bumetanide, must be interpreted within the limitations of the experimental design. The use of glucose as a sole substrate limited potential effects of exogenous fatty acids on the actions of bumetanide. Furthermore, insulin was not used in our preparations to mimic conditions of insulin deficiency. The presence of insulin may influence or alter the effects of bumetanide observed in this study. However, clinical studies suggest that the presence of insulin does not impact the efficacy of bumetanide or other loop diuretics. Because hyperglycemia has been shown to influence the severity of ischemic injury in a dog diabetes model (15), as well as in nondiabetic rat hearts (42), it is likely that we may have underestimated the severity of ischemic injury by maintaining a lower glucose concentration during perfusion.

In conclusion, the data from our experiments show that net inward flux through the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter activity is increased in diabetic hearts under normoxic conditions. Furthermore, Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter inhibition using bumetanide attenuates the ischemic rise in intracellular sodium, maintains higher levels of high-energy phosphates, reduces CK release, and significantly improves functional recovery after ischemia in diabetic, but not in nondiabetic hearts. These findings suggest a potential role for Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter inhibitors as novel therapeutic agents in the treatment of myocardial ischemia, specifically in diabetic patients.

R. Ramasamy was supported by an American Diabetes Association Career Development Award, an Established Investigator Award from the American Heart Association, by Juvenile Diabetes Foundation International Grant 196098, and by National Heart, Lung, and Blood Institute Grants HL-58408 and HL-61783.

**REFERENCES**


