Protection of ischemic myocardium in diabetics by inhibition of electroneutral Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter

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Diabetes in 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 diabetic, in part due to lack of complete understanding of the alterations in ion transport pathways in diabetics.

Of the data available, studies have shown that the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (32, 35, 43), Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (16, 17, 37), and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger are impaired in diabetic hearts (8, 43). The result of these changes in transporter activities is an increase in intracellular sodium under baseline conditions in diabetic hearts (12, 35, 37). A component of this increase may be an alteration in other sodium entry pathways, such as the electroneutral Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter in diabetic hearts. Our goal in this study was to address the following questions. First, is the net inward flux via the electroneutral Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter increased in a genetically Type I diabetic rat heart? Second, does this cotransporter contribute to increases in intracellular sodium during ischemia in diabetic hearts? Third, does inhibition of the electroneutral Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter limit injury and functional impairment in diabetic hearts during ischemia-reperfusion? We examined these questions in diabetic and nondiabetic hearts.
abetic rat hearts by inhibiting the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter with bumetanide and measuring radioac-

tive \(^{86}\)Rb uptake, intracellular sodium (by using \(^{23}\)Na spectroscopy), intracellular pH, and high-energy phos-

gphates (by using \(^{31}\)P NMR spectroscopy) during global ischemia-reperfusion. The data indicate that the flux

t via Na\(^+\)-K\(^+\)-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 Re-

search committee for Animal Care and Use at Columbia Univer-

sity (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 Massa-

chusetts Medical Center (Worcester, MA). The 3- to 4-mo-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 littersmates, also from the colony mainta-

ined 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 littersmates 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 (LVPD) was determined with the use of a

latex balloon in the left ventricle with high-pressure tubing

coupled to a pressure transducer. Perfusion pressure was

monitored with the use of high-pressure tubing off the perfusion line. Hemodynamic measurements were re-

corded on a four-channel Gould recorder. This design allowed

us to rapidly change perfusion media. The hearts were per-
fused 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 tem-

pered, 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 \(0.003 \times \text{arterial Po}_2 - 0.003 \times \text{effluent Po}_2\) \times \text{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 sev-

eral organs and cells [see the recent review by Russell (40)].

At concentrations of 10\(^-6\)–10\(^-7\) M, bumetanide (inhibitory

constant \(\sim 1 \times 10^{-6}\) 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 \(^{86}\)Rb Uptake via

Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter

Bumetanide-sensitive \(^{86}\)Rb (an analog of K\(^+\)) uptake was

measured by perfusing hearts with radioactive \(^{86}\)RbCl (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 \(^{86}\)RbCl buffer for 60 min to measure the

take of \(^{86}\)Rb by all K\(^+\) pathways. To measure the \(^{86}\)Rb

take via the bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotrans-

porter, diabetic (\(n = 6\)) and nondiabetic (\(n = 6\)) hearts were

perfused with the buffer containing \(^{86}\)RbCl and bumetanide

for 60 min. The uptake of \(^{86}\)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 per-

fused with the cold Krebs buffer (without any RbCl) for 2 min

before being counted to remove \(^{86}\)Rb from the vasculature.

Myocardial \(^{86}\)Rb uptake was quantified by normalizing for

perfuse 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 pre-

pared by differential centrifugation as previously described

(49). Membranes were analyzed by Western blot after separ-

ating samples on 7.5% Tricine gels and electrophoretic

transfer to polyvinylidene difluoride membrane (Immo-

bilon-P, Millipore). Equal amounts of protein (100 mg of

protein) were loaded from each sample (diabetic or nondi-

abetic hearts). The anti-Na-K-Cl cotransporter monoclonal

antibody (T4 antibody) (23) and enhanced chemilumines-

cence assay (ECL, Amersham) was used to detect the Na\(^+\)-

K\(^+\)-2Cl\(^-\) protein. The intensity of the bands in the Western

blots was 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 per-

centage of CK released on reperfusion was calculated as

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

This normalization of CK release enabled us to correct for

lower total myocardial CK content in the diabetic hearts.

NMR Spectroscopy

\(^3\)P NMR spectroscopy. All spectroscopy were performed on

an AMX 400 (Bruker) or Omega-300 (General Electric) ver-

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

Eight 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 fluid 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 heterologous systems, diabetic membranes showed a significant increase in the Na⁺-K⁺-2Cl⁻ cotransporter protein compared to the control membranes.

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.

Ischemia Protocol

Four groups of hearts, diabetic and nondiabetic, without and with bumetanide, were studied continuously with the use of ⁴⁰⁸¹P or ²³Na NMR spectroscopy. NMR spectra were obtained every 5 min during baseline, ischemia, and reperfusion along with simultaneous measurements of heart rate, LV end-diastolic pressure (LVEDP), and LVDP. All hearts were perfused at 12.5 ml/min before and after ischemia, without recirculating the buffer; reperfusion was performed for 60 min by using standard perfusate without bumetanide. The ischemic period of 20 min was initiated after the equilibration period (20 min) with the use of standard perfusate or perfusate also containing the Na⁺-K⁺-2Cl⁻ cotransporter inhibitor bumetanide (5 μM) for 10 min before ischemia.

RESULTS

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

Fig. 1. ⁸⁶RbCl uptake in nondiabetic (CON), diabetic (DM), bumetanide-perfused nondiabetic (CON-BUM), and bumetanide-perfused diabetic (DM-BUM) hearts (n = 6 in each group). Normalized Rb uptake is determined by dividing the tissue Rb radioactive counts by weight of heart and the mean count. *P < 0.05, DM-BUM hearts had significantly reduced ⁸⁶RbCl uptake than DM hearts.
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>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDP</td>
<td>10</td>
<td>108 ± 17</td>
<td>21 ± 15‡</td>
<td>34 ± 6‡</td>
</tr>
<tr>
<td>EDP</td>
<td>11</td>
<td>11 ± 3</td>
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<td>MVO₂</td>
<td></td>
<td>27.6 ± 2.9</td>
<td>22.9 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>DM + BUM</td>
<td>9</td>
<td>122 ± 21</td>
<td>38 ± 9 †</td>
<td>23 ± 2.7</td>
</tr>
<tr>
<td><strong>Nondiabetic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDP</td>
<td>12</td>
<td>115 ± 19</td>
<td>16 ± 19‡</td>
<td>74 ± 19‡</td>
</tr>
<tr>
<td>EDP</td>
<td>11</td>
<td>11 ± 4</td>
<td>56 ± 14‡</td>
<td>29 ± 6.7</td>
</tr>
<tr>
<td>MVO₂</td>
<td></td>
<td>29.2 ± 6.7</td>
<td>26.9 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>CON + BUM</td>
<td>11</td>
<td>111 ± 24</td>
<td>29 ± 23‡</td>
<td>57 ± 21‡</td>
</tr>
<tr>
<td>LVDP</td>
<td>11</td>
<td>11 ± 4</td>
<td>66 ± 18‡</td>
<td>57 ± 21‡</td>
</tr>
<tr>
<td>EDP</td>
<td></td>
<td>31.9 ± 6.8</td>
<td>26.7 ± 6.6</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 cmH₂O pressure); MVO₂, myocardial oxygen consumption (expressed as nmol·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, LVDP recovery during reperfusion was significantly greater in DM-BUM hearts vs. DM, CON, and CON + BUM hearts. §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>
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<tbody>
<tr>
<td><strong>Diabetic</strong></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>
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<tr>
<td>DM-BUM</td>
<td>9</td>
<td>756 ± 216</td>
<td>186 ± 36</td>
<td>24.6 ± 4.5*</td>
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<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; †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

²³Na 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
nondiabetic hearts, with the end-ischemic [Na]_i 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

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**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 P-31 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.
Increased Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} Cotransporter Flux and Intracellular Sodium in Diabetic Hearts

Steady-state [Na\textsubscript{i}] is maintained by a balance between sodium influx and efflux. Under baseline conditions, sodium influx occurs via the sodium channels, the Na\textsuperscript{+}-H\textsuperscript{+} exchanger, the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter, and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, whereas sodium efflux occurs mainly through the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (19). In hearts from diabetic animals, inhibition of the myocardial Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the presence of active sodium influx via other transporters results in an increased baseline [Na\textsubscript{i}] (18, 35, 43). In the current experiments, the baseline \textsuperscript{86}RbCl uptake measurements demonstrated an increased flux via the bumetanide-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter in diabetic hearts, findings that mirror a report (25) that demonstrated a 70% increase in flux via the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-}cotransporter in aortas from diabetic rats. The flux studies using \textsuperscript{86}Rb\textsuperscript{+} (K\textsuperscript{+} analog) and bumetanide indicate that the net flux via the bidirectional Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter is directed toward increasing intracellular ion concentration (Na, K, and Cl).

An increase in the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} 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\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter protein levels measured by western blots were similar in diabetic and nondiabetic hearts, indicating that the observed increases in the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter activity were likely due to activation by endogenous factors.

Increased Flux Via Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} Cotransporter and Intracellular Sodium During Ischemia

The changes in intracellular sodium during ischemia result from the balance of changes in Na\textsuperscript{+} 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\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter to the increase in intracellular sodium during ischemia in diabetic hearts. Two studies support our observations that the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter may play a greater role in the influx of sodium during ischemia when Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity is reduced. Anderson et al. (1) have shown that inhibition of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter with bumetanide decreased the intracellular Na\textsuperscript{+} 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>Intracellular pH at End of Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>7.01±0.04</td>
<td>6.04±0.04‡</td>
<td>6.79±0.06‡</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>
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<tr>
<td>Nondiabetic</td>
<td>7.03±0.02</td>
<td>5.95±0.06‡</td>
<td>7.02±0.08</td>
</tr>
<tr>
<td>CON</td>
<td>7.01±0.06</td>
<td>6.01±0.08‡</td>
<td>6.98±0.06</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>
</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.
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 \(\sim 50\%\) in diabetic hearts, while the reduction was \(\sim 20\%\) 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 diabetes 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.

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