The cyclic nucleotide cyclic adenosine monophosphate (cAMP) is an important second messenger in cell function. The intracellular concentration of cAMP is regulated by the opposing activities of adenylate cyclase, which is responsible for the synthesis of cAMP, and of cAMP phosphodiesterase (PDE), which catalyzes its degradation. PDEs are a family of enzymes which hydrolyze the 3'-ribose phosphate bond of the naturally occurring second messenger nucleotide 3'5'-cyclic monophosphate to form the inert 5'-nucleotide monophosphate. PDEs are growing groups of enzymes, classified into at least 7 families (PDE1 - PDE7) with several subtypes and splice variants that differ in their tissue distribution.

In Vivo Effects of Phosphodiesterase III Inhibitors on Glucose Metabolism and Insulin Sensitivity

**Background.** Milrinone is a widely-used phosphodiesterase III (PDE3)-selective inhibitor. Recently, it was shown that milrinone increased hepatocyte glucose production, glycerol release, and insulin secretion and fatty acid oxidation in vitro. Considering these results, it is important to know whether milrinone can induce insulin resistance in vivo.

**Methods.** In order to investigate the effects of a selective PDE3 inhibitor on insulin levels and dose-dependent relationship, varying doses of milrinone were injected into the right internal jugular vein in conscious rats, and blood glucose, plasma free fatty acid (FFA) and insulin levels were observed. The effects of milrinone on glucose metabolism and insulin sensitivity were assessed by using a hyperinsulinemic-euglycemic clamp.

**Results.** Chronically catheterized nonstressed rats were administered varying doses of milrinone (1, 5, 25 µmol/kg) and were compared with controls not treated with milrinone. After dosing, plasma FFA levels in the 3 milrinone groups significantly increased compared with before dosing and the controls. The percentages of elevation of FFA by the different milrinone doses were very similar, 50%, 52%, 55% for 1, 5, and 25 µmol/kg, respectively, at 2 min after dosing, suggesting that lipolysis is very sensitive to the effect of milrinone. However, the effect of milrinone on glucose concentration was detectable only in the 25 µmol/kg group, and the plasma insulin levels were significantly elevated in the 5 and 25 µmol/kg milrinone groups, indicating that there was a dose-response relationship between milrinone and insulin and glucose levels. During hyperinsulinemic clamp, there was a significant increase in plasma FFA (173.1 ± 15.2 to 633.8 ± 87.3 µEq/L, p < 0.01) and a significant decrease in glucose infusion rates (GIR) to about 21%, and a slight increase in plasma insulin after milrinone treatment.

**Conclusions.** These data suggest that milrinone impaired the abilities of insulin to suppress lipolysis and insulin-mediated glucose utilization in peripheral tissue, despite having an increase in insulin secretion. Therefore, we conclude that milrinone administration induces an acute increase in insulin resistance in vivo, and that may limit the therapeutic value of milrinone for human diabetic subjects.
PDE7 appear to be the most important in the regulation of cAMP. It is well known that elevated intracellular levels of cAMP potentiate insulin secretion in a glucose-dependent manner. How ever, only the inhibition of PDE3 increased glucose-stimulated insulin secretion from rat islets in vitro, leading to the conclusion that this isoform may play a major role in regulating insulin secretion.

Milrinone [2-methyl-5-cyano(3,4′-bipyridin)-6(1H)-one] is a widely-used PDE3-selective inhibitor. Recently it was shown that milrinone increased hepatic glucose production, glycerol release, insulin secretion and fatty acid oxidation in vitro. Con sidering these results, it is important to know whether milrinone can induce insulin resistance in vivo. In the present study, we examined the in vivo effects of milrinone on insulin secretion and lipolysis levels and dose-response relationship in conscious rats. We also assessed possible effects of milrinone on glucose metabolism and insulin sensitivity by using hyperinsulinemic-euglycemic clamping. This enabled us to evaluate the potential utility of milrinone for the treatment of diabetes.

**METHODS**

**Preparation of animals**

Male Sprague-Dawley rats weighing between 250 and 300 g were purchased from Charles River Laboratories (Wilmington, MA) and housed in an environment mentally controlled room with a 12-hour light/dark cycle, where they had free access to standard rat chow and water. Five to 7 days before the in vivo study, rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt). A silastic catheter (I.D. = 0.02 in) was inserted into the right internal jugular vein and extended to the level of the right atrium. The catheter for carotid artery was constructed from a short (25 mm) segment of polyethylene tubing (PE-10), connected to a 10-cm length of PE-50 by heat in a flame. The small end was advanced through the left carotid artery until its tip reached the aortic arch. The free ends of the catheters were attached to long segments of steel tubing and tunneled subcutaneously around the side of the neck to the back of the neck, where they were exteriorized through a skin incision and then secured anorectally to the skin by a standard wound clip. At the end of the procedure, catheters were flushed with 300 µl isotonic saline containing heparin (40 U/mL) and ampicillin (5 mg/mL) and then filled with a viscous solution of heparin (300 U) and 80% polyvinylpyrrolidone (PVP-10, Fisher, NJ) to prevent refluxing of blood into the catheter lumen.

**Protocol 1: Sampling procedure in vivo with out clamping**

Animals were allowed at least 5 days to recover from the effects of surgery. All studies were conducted in the morning following a 14-hour overnight fast. Throughout the study, the rats were allowed to move freely within the confines of their cages. All substrates were administered into the arterial catheter and blood samples were obtained from the venous catheters. Plasma samples for determination of plasma glucose, insulin and free fatty acids (FFA) concentrations were obtained at -30, -15, 0, 2, 15, 30, and 60 min during the study. Each blood sample was replaced by the same volume of fresh whole blood obtained by from litter mates of the test animal to prevent intravascular volume depletion and anemia. Four groups of rats were studied. In the first group (Group I, n = 6), 25% dimethyl sulfoxide (DMSO, Fisher) was injected at 0 time to serve as a control. In the remaining 3 groups, the varying doses of milrinone (Biomol Research Labs, Inc, MA) were administered at 0 time: Group II, 1 µmol/kg (n = 4); Group III, 5 µmol/kg (n = 5); Group IV, 25 µmol/kg (n = 6).

**Protocol 2: Sampling procedure with euglycemic clamping study**

The hyperinsulinemic euglycemic clamping technique was used. For all animals, a constant infusion of insulin (4.8 mU/kg.min) was added to maintain the plasma insulin concentration at the desired level. In the mean time, a variable infusion of 25% glucose was started and blood glucose level adjusted every 5–10 min with an Elite glucometer (Bayer, Inc, Japan) to clamp the plasma glucose concentration at fasting levels. At time 120, milrinone (25 µmol/kg in 25% DMSO) was injected respectively into the control group (n = 11) and milrinone group (n = 10). Plasma samples
Table 1. Blood and plasma parameters in the control and milrinone-treated rats in protocol 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group(^a)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5.0 ± 0.2</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>II</td>
<td>4.7 ± 0.2</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>III</td>
<td>4.6 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>IV</td>
<td>4.7 ± 0.1</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>FFA (µEq/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>668.6 ± 34.6</td>
<td>671.7 ± 25.1</td>
</tr>
<tr>
<td>II</td>
<td>726.6 ± 24.1</td>
<td>654.4 ± 39.4</td>
</tr>
<tr>
<td>III</td>
<td>667.1 ± 51.2</td>
<td>577.0 ± 41.4</td>
</tr>
<tr>
<td>IV</td>
<td>675.6 ± 41.1</td>
<td>656.9 ± 61.9</td>
</tr>
<tr>
<td>Insulin (pmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1113 ± 26.0</td>
<td>1150 ± 22.9</td>
</tr>
<tr>
<td>II</td>
<td>881.1 ± 13.4</td>
<td>790.0 ± 11.9</td>
</tr>
<tr>
<td>III</td>
<td>846.6 ± 6.97</td>
<td>832.2 ± 5.2</td>
</tr>
<tr>
<td>IV</td>
<td>1315.8 ± 28.9</td>
<td>975.4 ± 24.0</td>
</tr>
</tbody>
</table>

\(^a\)Group I received 25% DMSO alone; the dosages of milrinone in Group II, III and III are 1 µmol/kg, 5 µmol/kg and 25 µmol/kg, respectively; \(^b\) \(p < 0.05\), \(^c\) \(p < 0.01\) vs. Group I; \(^d\) \(p < 0.05\), \(^e\) \(p < 0.01\) vs. basal; FFA = free fatty acids.

for determination of plasma glucose, insulin and free fatty acids (FFA) and concentrations were obtained at -90, 0, 100, 120, 130, 140, 150, 160 and 180 min during the study. Each blood sample was replaced by the same volume of fresh whole blood as above.

**Analytical procedures**

Plasma in su lin was measured by radioimmunoassay (RIA) using rat in su lin as standard (Linco Re search, Inc. MO), and its inter- and intra-assay CV% were 3.5% and 3.8%, respectively. Enzymatic colorimetric kits were used to determine plasma con centration of FFA (Wako Chemicals, Inc. VA). The inter-and intra-assay CV% were 2.5% and 2.9%, respectively, during measurement of plasma FFA.

**Statistical analysis**

Data are presented as the mean ± SE. Comparisons between groups were made by rank sum tests. All statistical analyses were performed using SPSS.

**RESULTS**

**Pro to col 1. Study with out clamp ing**

The characteristics before and after milrinone or DMSO treatments are shown in Table 1. The plasma FFA concentrations before milrinone or DMSO treatments were similar for the control and the 3 milrinone groups. After dosing, plasma FFA levels in the 3 milrinone groups significantly increased compared with before dosing and the controls. Two min after administration of milrinone, the per cent ages of elevation of FFA in Group II, III and IV were 50.3%, 52.3%, and 54.9% respectively. On the other hand, the effect of milrinone on lipolysis was very rapid (Table 1). Within 2 min utes, the plasma FFA were elevated by all 3 different milrinone doses (Group II: 654.4 ± 39.4 to 983.2 ± 63.1 µEq/L, \(p < 0.05\); Group III: 577.0 ± 41.4 to 879.3 ± 36.8 µEq/L, \(p < 0.05\); Group IV: 656.9 ± 61.9 to 1017.8 ± 107.1 µEq/L, \(p < 0.01\) for 25 µmol/kg; Table 1). In Group IV, the elevation of FFA lasted at least 15 min utes and then declined gradually and tailed out beyond 60 min. In contrast, the lower doses of milrinone elicited shorter durations of elevation (Table 1). The effect of milrinone on blood glu cose con centration was detectable only in Group IV that an elevation of plasma glu cose con centration from 4.6 ± 0.2 mmol/L before milrinone treatment to 6.1 ± 0.4 mmol/L (\(p < 0.05\)) 15 min after treatment, and it continued beyond 60 min after the dosing (Table 1). In Groups 2 and 3, the lower milrinone doses did not elicit a sig nificant rise in plasma glu cose. The plasma in su lin lev els were sig nificant el e-
vated in Groups 3 and 4 ($p < 0.05$ and $p < 0.01$, respectively). The elevation in Group IV was more than 3 times the basal level at 15 min after administration ($448.2 \pm 61.2$ vs. $102.8 \pm 10.9$ pmol, $p < 0.01$). There was a slight elevation of insulin concentration in Group II at 2 min after dosing, but no statistical significance was found (Table 1).

### Protocol 2. Insulin clamping study

The elevation of plasma FFA, glucose and insulin induced by milrinone was an interesting observation. Our next goal was to find out if milrinone induces insulin resistance by using euglycemic-hyperinsulinemic clamping. High-dose milrinone (25 $\mu$mol/kg) was used for these studies. The characteristics before and after milrinone administration are shown in Table 2 and Fig. 1 and 2. At 120 min after the start of the clamping, milrinone was administered usually through the jugular vein catheter at a slow rate (300 $\mu$l/60 seconds). Glucose infusion rates (GIR) were around 26 mg/kg/min until milrinone was administered (Table 2). Then there was a decrease in GIR immediately (to about 21%), while the DMSO treatment did not affect the GIR. The decrease was not recovered for at least 60 min (Fig. 1C). In the meantime, there was a significant elevation of plasma insulin in the milrinone group 10 min after dosing (from $442.4 \pm 40.5$ to $591.1 \pm 83.3$ pmol, $p < 0.05$, about 37% increase, Fig. 2B).

In insulin fusion in hyperinsulinemic clamping inhibits lipolysis. The FFA levels decreased for both the control and the milrinone groups. At 120 min, the FFA levels were decreased from $759.8 \pm 59.0$ to $187.4 \pm 12.8$ $\mu$Eq/L for the controls and from $719.3 \pm 73.3$ to $173.1 \pm 15.2$ $\mu$Eq/L for the milrinone group, a decrease of more than 76% for both groups. The decrease was maintained by the hyperinsulinemic controls, while milrinone rapidly and briefly raised the FFA concentration to $633.8 \pm 87.3$ $\mu$Eq/L at 10 min after dosing ($p < 0.01$ vs. control and 120 min). Then it decreased to very close to the control level at about 40 min (Fig. 2A).

### DISCUSSION

In the Protocol 1 studies, we showed that milrinone 25 $\mu$mol/kg induced an acute increase in plasma glucose
and FFA levels. The 2 low-dose milrinone groups also induced acute increases in plasma FFA, but not glucose. The percentages of elevation of lipolysis by the different milrinone doses were very similar, 50.3%, 52.4% and 54.9% for 1, 5 and 25 µmol/kg, respectively, at 2 minutes after administration. The elevation induced by the 25-µmol/kg dose lasted at least 15 minutes and then declined gradually. The lower doses of 1 and 5 µmol/kg elicited a shorter duration of elevation. These findings suggest that the effect of milrinone on lipolysis is very sensitive and rapid, and confirm and extend previous observations that milrinone increased glycerol release from adipocytes, implying milrinone stimulated lipolysis, presumably via cAMP-mediated activation of hormone-sensitive lipase.\textsuperscript{10,11} Although increased lipolysis would tend to increase circulating glucose levels by liberating fatty acids that could serve as an alternate fuel source, only an approximate 2-fold and prolonged elevation of plasma FFA levels in duced by 25 µmol/kg dose resulted in a time-dependent elevation of plasma glucose level. As shown in Table 1, the plasma inulin concentrations were elevated significantly in the 5, 25 µmol/kg milrinone groups, not in the 1 µmol/kg group. These suggest that there was a dose-response relationship between milrinone and inulin and glucose levels. We know that the role of cAMP in regulating insulin secretion is quite well-established. The intracellular levels of cAMP are regulated by the opposing activities of adenylate cyclase, which is responsible for the synthesis of cAMP, and PDE, which catalyzes its degradation. And there is evidence to suggest the PDE isoform that modulates inulin secretion from the pancreatic cell in vitro belongs to the PDE3 family, and insulin secretion is stimulated by PDE3-selective inhibitors such as milrinone while inhibitors selective for other PDE isoforms are ineffective, and the potentiated insulin secretion is in a glucose-dependent manner.\textsuperscript{12,13} But in our results, milrinone at 5 µmol/kg induced a significant elevation in insulin secretion, glu cose concentration was not changed. So we may therefore consider cAMP as a positive modulator of hormone secretion, rather than a primary trigger of insulin release. On the other hand, FFA are also strong insulin secretagogues, and acute stimulation of insulin secretion by FFA has been well established in humans and animals.\textsuperscript{14}

In order to further examine the role of milrinone in insulin-mediated carbohydrate metabolism and the insulin sensitivity of peripheral tissue, the hyperinsulinemic-
euglycemic clamp ing technique was used. In our hy -
perinsulinemic clamp ing, in su lin fu sion in creased
plasma in su lin lev els and fur ther in hib its lipolysis. In our
study, the FFA con cen tra tions de creased for both the
con trol and the milrinone group. Af ter milrinone ad -
im is tra tion, the FFA level had a rapid and brief in crease
more than 3.5-fold over basal lev els (Ta ble 2, Fig. 2A),
sug gest ing milrinone im paired the abil ity of in su lin to
sup press lipolysis. Un der phys i o log i cal con di tions lipid
mo bi li za tion from ad i pose tis sue is tightly con trolled by
the adrenergic sys tem and some lo cal mod u la tors such as
adenosine and prostaglandins.15 Milrinone in hib its PDE
activity and hinders the response to endogenous
adenosine in var i ous tis sues. In fact, cAMP for ma tion in
fat cells is an in ter med i ate step in the ac ti va tion of
triglyceride lipase by hor mones and neurotransmitters
interacting with stimulatory receptors.10 Dur ing our
hyperinsulinaemic-clamping study, plasma insulin lev -
els did not dif fer sig nif i cantly be tween the con trol and
milrinone groups af ter milrinone treat ment, but there
was a slight sig nif i cant el e va tion when com par ing the
time point be fore milrinone ad min is tra tion (Ta ble 2, Fig.
2B). A pos si ble ex pla na tion is that en dog e nous in -
sulin se cre tion is slightly sup pressed by the in fu sion of ex og e-

**Fig. 2.** Plasma levels of free fatty acids (FFA) (A) and insulin (B) and glucose infusion rates (GIR) (C) during
hyperinsulinemic-euglycemic clamping. Milrinone injection (25 mol/kg.min) at time 120. *p < 0.05, **p < 0.01 vs. controls; ♦p < 0.05, ♦♦p < 0.01 vs. 120 min).
nous in insulin. How ever, there was a sig ni ficant de crease in GIR in the milrinone group af ter treat ment. The de crease was not recovered for at least 40 minutes. This sug gests that milrinone can be con trib uted to a de crease in glu cose uti li za tion in pe riph eral tis sues, and/or an in crease in endoge nous glu cose pro duction (EGP), in dicat ing it im pairs the glu co reg u latory ac tion of in su lin. The mech a nism of this de crease re mains poorly un der stood and may in volve dif fer ent reg u la tory steps. A pos si ble ex pla na tion that has been pro posed is an in crease in the ox i da tion of fatty ac ids by stim u lat ing the up take of ex tra cellu lar FFA via AL-adenosine re ceptors. The end products of fatty acid β-ox i da tion such as acetyl-CoA and NADH in hibit the pyruvate dehydrogenase com plex (PDH) to lead to the de crease in glu cose uti li za tion by periph er al tis sues, and pro mote glu co neo gen e sis. On the other hand, it has been shown that the el eva tion of plasma levels of FFA pro du ces at least 2 dis tinct bio chem i cal de fects: 1) in hibi tion of in su lin-stim ulated glu cose trans port and/or phos phory la tion; and 2) in hi bi tion of muscle gly co gen syn the sis ac tivity.

In summary, our results sug gest that milrinone an ta gon izes the abil ity of in su lin to pro mo te to tal-body glu cose uptake and sup press lipolysis during hy perin su lin e mic- eugly cemic clamp ing, de spite hav ing a slight in crease in in su lin se cre tion. PDE3B may play an im por tant role in the anti lipolytic ac tion of in su lin and the re lease of fatty ac ids from adipo cytes. In creased con cen tra tions of circu lat ing fatty ac ids as a re sult of ele vated or unrestrained lipolysis cause insulin resis tance. There fore, we con sider it is likely that milrinone ad min istered in acute in in su lin re sis tance in vivo, and that may limit the the ra peu tic value of milrinone for hu man di a be tic sub jects.

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