Metoprolol improves cardiac function and modulates cardiac metabolism in the streptozotocin (STZ) diabetic rat.

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Abstract

The effects of diabetes on heart function may be initiated or compounded by the exaggerated reliance of the diabetic heart on fatty acids and ketones as metabolic fuels. β-blocking agents such as metoprolol have been proposed to inhibit fatty acid oxidation. We hypothesized that metoprolol would improve cardiac function by inhibiting fatty acid oxidation and promoting a compensatory increase in glucose utilization. We measured \textit{ex vivo} cardiac function and substrate utilization following chronic metoprolol treatment and acute metoprolol perfusion. Chronic metoprolol treatment attenuated the development of cardiac dysfunction in the STZ-diabetic rats. Following chronic treatment with metoprolol, palmitate oxidation was increased in control hearts but decreased in diabetic hearts without affecting myocardial energetics. Acute treatment with metoprolol during heart perfusions led to reduced rates of palmitate oxidation, stimulation of glucose oxidation and increased tissue ATP levels. Metoprolol lowered malonyl CoA levels in control hearts only, but no changes in ACC phosphorylation or AMPK activity were observed. Both acute metoprolol perfusion and chronic in vivo metoprolol treatment led to decreased maximum activity and decreased sensitivity of CPT-1 to malonyl CoA. Metoprolol also increased SERCA expression and prevented the re-expression of ANP in diabetic hearts. These data demonstrate that metoprolol ameliorates diabetic cardiomyopathy and inhibits fatty acid oxidation in STZ-induced diabetes. Since malonyl CoA levels are not increased, the reduction in total CPT-1 activity is the most likely factor to explain the decrease in fatty acid oxidation. The metabolism changes occur in parallel with changes in gene expression.

\textbf{Keywords:} Diabetic cardiomyopathy, fatty acid oxidation, heart failure, carnitine palmitoyltransferase, malonyl coenzyme A.
**Introduction**

Diabetes is an independent risk factor for cardiovascular death, and mortality following myocardial infarction is increased in diabetic patients (1, 2, 16, 36). The most common cause of this cardiovascular mortality is heart failure arising as a result of hypertensive or ischemic injury. However, the diabetic heart is rendered more susceptible to these injuries as a result of a number of pathological changes which are collectively referred to as ‘diabetic cardiomyopathy’. These include cell death, oxidative stress, impaired calcium handling and decreased calcium sensitivity of myofilaments, alterations in second messenger signaling pathways (RhoA/ Rho Kinase, protein kinase C), induction of the ‘fetal gene program’ and changes in cardiac metabolism (17, 26, 34). The diabetic heart, with marked limitations on the catabolic use of glucose, relies heavily on alternative fuels such as fatty acids and ketone bodies, resulting in the accumulation of intermediates and products of fatty acid and glucose metabolism, as well as the accumulation of intramyocellular glycogen and triglycerides (15, 23). These changes are potentially harmful to the cardiomyocyte, and agents which restore the normal balance of cardiac substrate utilization by inhibiting fatty acid oxidation have been shown to improve cardiac function in diabetic hearts (42).

The β-blockers metoprolol (β1 selective inverse agonist), bisoprolol (β1 selective antagonist), nebivolol (β1 selective antagonist) and carvedilol (non-selective β and α1-antagonist) reduce mortality and improve cardiac function in heart failure patients (18, 24). Putative mechanisms include antiarrhythmic effects, amelioration of cardiomyocyte hypertrophy, necrosis and apoptosis, reversal of fetal gene program expression, increases in cardiac receptor density (for some β-blockers including metoprolol) and modulation of cardiac metabolism (see (31) for review). To date, metoprolol (21), bucindolol and carvedilol (3) have been shown to inhibit fatty acid oxidation, although it is not clear to
what extent these effects are due to direct effects on the heart or alterations in substrate supply mediated through effects on peripheral organs. Carvedilol is a non-selective β-blocker which also blocks the α1-adrenergic receptor, calcium channels (at high doses) and is an antioxidant (22). Metoprolol is a selective inverse agonist of the β1 receptor which is likely to also block β2 receptors at clinical doses (31). Clinical studies have shown that treatment with metoprolol decreases fatty acid oxidation and promotes glucose oxidation in patients with dilated cardiomyopathy (21). A study in dogs with microembolism-induced heart failure showed that the enzyme carnitine palmitoyltransferase-1 (CPT-1) is inhibited by chronic metoprolol treatment (37). CPT-1 catalyzes the entry of long chain fatty acids into the mitochondria, a critically important control step in fatty acid oxidation.

Given the broad range of actions attributed to β-blockers, and their demonstrated benefit in other forms of heart failure, we hypothesised that these drugs would improve cardiac function in diabetic cardiomyopathy. Here we have investigated both the acute and chronic effects of metoprolol on cardiac function and cardiac substrate utilization in the streptozotocin (STZ) diabetic rat. The streptozotocin (STZ) diabetic rat is a model of poorly controlled type 1 diabetes which is associated with a marked decrease in insulin levels. STZ is an antibiotic synthesised by the bacterium Streptomyces achromogenes which selectively targets and destroys the insulin-secreting β-cells of the pancreas (27, 43). The diabetic cardiomyopathy of the STZ rat closely resembles that which is seen clinically, and, at the STZ dose used in our laboratory (60mg/ kg), appears 6 weeks following STZ injection (45, 47, 48). STZ rats do not develop atherosclerosis or hypertension, thereby enabling the diabetic heart to be studied in the absence of ischemic or hypertensive disease.
The aim of the present investigation was to provide proof of concept that β-blockers can improve cardiac function in the diabetic heart, and to investigate whether inhibition of fatty acid oxidation contributes to this benefit. We chose to focus the present study on a single β-blocker, metoprolol, because it has previously been shown to alter cardiac energy substrate utilization by the heart, and because this action has been linked to inhibition of CPT-1 activity. Furthermore, the actions of metoprolol are more selective than those of carvedilol. We hypothesized that the β-blocker metoprolol improves function in the diabetic heart. Inhibition of CPT-1 by metoprolol would allow the heart to utilize glucose and could contribute to the improvement in cardiac function by relieving the injury caused by the switch in substrate selection.

Materials and Methods

Animal Model and Treatments

Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Male Wistar Rats (weight matched 200-220g) were purchased from Charles River Laboratories and allowed to acclimatize for 1 week prior to the beginning of the study. Rats were allowed ad libitum access to standard rat chow and water. For the preliminary cardiac function study, rats were randomly divided into four groups: control (C), control treated (CT), diabetic (D) and diabetic treated (DT). In the cardiac metabolism studies, two additional groups were added: control perfused (CP) and diabetic perfused (DP). Diabetes was induced by the injection of 60mg/kg streptozotocin (STZ) into the caudal vein. One week following the induction of diabetes, treatment was commenced. The treated groups received 75 mg/ kg/ day metoprolol by intraperitoneal injection while untreated groups received an equivalent volume of vehicle. This dose, equivalent to a daily human dose of 100 mg per day
(correcting for inter-species differences in surface area/volume ratio), was well tolerated by the rats in preliminary studies, and produced a significant improvement in cardiac function in the diabetic treated group. Six weeks following the induction of diabetes, the animals were euthanized. 5-hour fasting blood samples were taken one week following STZ-injection and immediately prior to termination. For perfused groups, metoprolol was added to the perfusate in the isolated working heart preparation as described below.

**Measurement of Plasma Parameters**

Plasma glucose concentration was determined using the Beckmann Glucose analyzer. Plasma insulin was measured using the radioimmunoassay kit available from Millipore/LINCO (Billerica, Massachusetts). Plasma free fatty acids, cholesterol and triglycerides were determined by colorimetric assay kits available from Roche (Basel, Switzerland). Plasma ketone levels were measured using the CardioChek Analyzer from Polymer Technology Systems, Inc (Indianapolis, Indiana).

**Measurement of Cardiac Function and Metabolism**

Measurement of cardiac function and metabolism was carried out as previously described (6, 10). Six weeks after STZ injection, the rats were anesthetized by 4% isoflurane anesthesia and the hearts excised. The hearts were perfused with Krebs buffer (composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 5.5 mM glucose, 0.5 mM lactate, 100 or 0 μunits/ml insulin, 0.8 mM palmitate bound to 3% BSA) in an aerobic perfusion for 60 minutes. These perfusion conditions were selected to maintain consistency with those used previously by our laboratory and others for measurements of this kind (4, 7, 20, 29). Since plasma lipid levels did not vary between
groups, a palmitate concentration of 0.8 mM palmitate was used in all groups; although the plasma fatty acid level was approximately 0.2 mM, a significant proportion of the fatty acid supply to the heart \textit{in vivo} comes from plasma lipoproteins (9, 35, 41), so a higher concentration of palmitate was selected.

For simultaneous measurement of glucose and palmitate oxidation, the production of $^{14}$CO$_2$, H$^{14}$CO$_3^-$ and $^3$H$_2$O from $^{14}$C glucose and $^3$H palmitate was measured at 10 minute intervals. Lactate production was determined by measuring the net accumulation of lactate in the perfusate at the same time intervals. Cardiac output, aortic and pulmonary flow were measured by probes positioned upstream of the pulmonary cannula and downstream of the aortic cannula throughout the course of the perfusion. Pressure was measured by a pressure transducer positioned downstream of the aortic cannula. For perfused groups (CP, DP), 2000 ng/ ml (4.8 $\mu$M) metoprolol was added to the perfusate after 30-minutes. For other groups (C, CT, D, DT), an equivalent volume of vehicle was added. Following completion of the perfusion, tissues were freeze clamped in liquid nitrogen, weighed and stored at -70°C for further assay. The n-numbers were 5 for all groups. Biochemical assays and western blots were only carried out on samples which had been perfused with insulin.

**Biochemical Assays**

Tissue triglyceride levels were measured as previously described (14). Enzyme activities were measured on whole tissue homogenates. Total protein concentration was measured using the Bradford assay. CPT-1 activity was measured by measuring the rate of conversion of $^{14}$C carnitine to $^{14}$C acyl carnitine as previously described (13). The sensitivity of CPT-1 to malonyl CoA was measured by assaying CPT-1 activity in the presence of increasing concentrations of malonyl CoA and calculating an IC$_{50}$ value (28). To test for pharmacological inhibition of CPT-1 by metoprolol, whole
homogenates of control hearts were incubated with increasing concentrations of metoprolol in the presence of 0, 50 or 100 µM malonyl CoA.

AMPK was purified by immunoprecipitation prior to assay, based on the rate of incorporation of $^{32}$P from [$^{32}$P]ATP into a synthetic peptide containing a specific AMPK consensus sequence (AMARAASAAALARRR), using the kit from Upstate Biotechnology) (5). ATP, ADP, AMP and malonyl CoA levels were measured in tissue samples that had been snap-frozen and extracted with perchloric prior to HPLC analyzes as previously described (8) (30). Active pyruvate dehydrogenase complex (PDC) activity was assayed by measuring the rate of conversion of sodium pyruvate and coenzyme A to acetyl CoA in the presence of NaF as previously described (33). PDC expression was measured as an index of total PDC levels.

**Western Blot Analysis and ELISA**

Whole tissue homogenates were prepared from frozen and powdered heart tissue in a lysis buffer as previously described (33). Samples of homogenates (~100µg protein) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to probe for acetyl CoA carboxylase (ACC, 1:500 dilution, Upstate Biotechnology/ Millipore, Billerica, Massachussets), phospho-ACC (Ser79, 1:1000 dilution, Upstate Biotechnology, Billerica, Massachussets), pyruvate dehydrogenase complex E2 (1:1000 dilution, Santa Cruz biotechnology, Santa Cruz, California), sarcoplasmic reticulum calcium ATPase (SERCA, Upstate Biotechnology/ Millipore), $\alpha$-myosin heavy chain ($\alpha$-MHC, Upstate Biotechnology/ Millipore) and malonyl CoA decarboxylase (1:1000, a generous gift from J. Dyck and G.D. Lopaschuk, University of Alberta). To measure the total phosphorylation state of ACC, samples underwent immunoprecipitation with anti-phosphoserine or
anti-phosphothreonine antibodies (Upstate Biotechnology/ Millipore) prior to SDS-PAGE and immunoblotting for ACC. Tissue levels of atrial natriuretic protein (ANP) were determined by measuring pro-ANP levels using the ELISA kit from Biomedica (Wien, Austria).

**Data Analysis**

Data are expressed as mean ± standard error of the mean (SEM). For statistical analysis, data were analyzed using Number Cruncher Statistical Software (NCSS, Kaysville, Utah). Starling curves were analyzed using GLM ANOVA with Neumann-Keuls post hoc test. All other data were analyzed using One-Way ANOVA with Neumann-Keuls post hoc test. Acute perfusion and chronic treatment data are presented separately, although the control and diabetic groups for each are the same, and the data were subjected to composite analysis. Metabolic rates and tissue metabolite levels were expressed per g of wet or dry weight whereas enzyme activity was expressed per mg protein; interpretation of the data is not affected by these differences, as these methods are designed to reveal patterns of changes.

**Results**

**Plasma Parameters and General Characteristics**

Plasma glucose and insulin levels were measured to confirm successful induction of diabetes; as expected, neither were affected by chronic metoprolol treatment (Table 1). Plasma lipids were mildly elevated in the diabetic group and metoprolol treatment did not lead to significant changes in either control or diabetic animals. Surprisingly, metoprolol ameliorated the increase in ketone levels in the diabetic group (Table 1). As expected, body weights of diabetic animals were lower than those of
controls and metoprolol treatment had no significant effect on body weight of either group. However, metoprolol treatment led to significantly lowered heart weight in both control and diabetic rats (Table 1). The heart weight to body weight ratio was only decreased by metoprolol in control hearts.

**Effects of Chronic In Vivo Metoprolol Treatment on Metabolism of Isolated Perfused Hearts**

Chronic metoprolol treatment ameliorated the depression in hydraulic power in the diabetic group (Figure 1). A similar pattern was observed for rate-pressure product and cardiac output; the improvement in rate-pressure product was attributable to an increase in heart rate rather than an increase in peak systolic pressure.

We next studied the effects of metoprolol on glucose and palmitate oxidation. Chronic treatment with metoprolol decreased glucose oxidation by 43% and increased palmitate oxidation by 126% in control hearts (Figure 2A). Glycogen levels were decreased by 47% but lactate production was unchanged. In the diabetic hearts, palmitate oxidation was increased by 333% relative to controls and glucose oxidation was negligible; in these hearts, chronic metoprolol treatment decreased palmitate oxidation by 39% and increased glucose oxidation by 80% (Figure 2A). Glycogen levels were elevated in diabetic hearts as compared with controls and neither glycogen levels nor lactate production (as determined by lactate accumulation in the perfusate) were affected by metoprolol treatment (Table 4). Tissue triglyceride levels were increased by 44% in the diabetic group. In both control and diabetic hearts, metoprolol treatment lowered tissue triglyceride levels by 25% (Table 4). It is important to note that glycogen and triglyceride levels were measured at the end of the perfusion. Myocardial energetics, as determined by tissue levels of ATP, ADP and AMP, and AMPK activity, were not altered either by metoprolol or by diabetes (Table 2). When perfusions were repeated in the absence of insulin, the effect of metoprolol on glucose oxidation was obliterated (glucose oxidation: C
421 ± 66, CT 385 ± 59, D 54 ± 53*, DT 47 ± 25* nmol/ min/ g dry weight, * significantly different from C and CT, p<0.05), but the effect on palmitate oxidation was preserved (palmitate oxidation: C 1126 ± 10.9, CT 1649 ± 155*, D 2490 ± 921+, DT 1777 ± 175* nmol/ min/ g dry weight, * significantly different from untreated group, + significantly different from all groups, p<0.05).

**Acute Effects of Metoprolol on Metabolism of Isolated perfused Hearts**

Perfusion of hearts with metoprolol for 30 minutes resulted in 42 and 46% inhibition of palmitate oxidation with 177% and 237% stimulation of glucose oxidation in control and diabetic hearts respectively (Figure 3A). ATP levels increased by 15% and 62% whereas AMP levels decreased by 70% and 47% in control and diabetic hearts respectively (Table 3). In diabetic hearts, acute metoprolol perfusion also raised ADP levels by 32% (Table 3). Lactate production was decreased by 35% and 77% in control and diabetic hearts respectively following acute metoprolol perfusion, but glycogen levels were unchanged (Figure 3, Table 4). In both control and diabetic hearts, acute metoprolol perfusion lowered tissue triglyceride levels by 15% and 47% respectively (Table 4). When the perfusions were repeated in the absence of insulin, the effect of metoprolol on glucose oxidation was obliterated in diabetic hearts but not control hearts (glucose oxidation: C 421 ± 66, CP 967 ± 66 +, D 54 ± 53*, DP 52 ± 51* nmol/ min/ g dry weight, * significantly different from C and CT, + significantly different from all groups p<0.05), whereas the pattern of changes observed for palmitate oxidation was preserved (palmitate oxidation: C 1126 ± 10.9, CP 770 ± 102*, D 2490 ± 921+, DP 1019 ± 242* nmol/ min/ g dry weight, * significantly different from untreated group, + significantly different from all groups, p<0.05).

**Regulation of Myocardial Malonyl CoA Concentrations**
Myocardial malonyl CoA levels were lowered by 55% following chronic in vivo treatment with metoprolol and were also lowered acutely by 53% when hearts were treated with the beta-blocker during perfusion (Table 4). Chronic metoprolol treatment did not alter ACC or MCD expression (Table 4); neither was AMPK-mediated phosphorylation of ACC altered, as assessed by phosphorylation of Ser 79 on ACC, either during metoprolol perfusion or chronic in vivo metoprolol treatment (Figure 5). Furthermore, the total phosphorylation state of ACC was also unaffected by metoprolol, based on reactivity with pan-specific anti-phosphoserine and anti-phosphothreonine antibodies. Overall, malonyl CoA levels did not correlate with the observed changes in the rate of fatty acid oxidation, and the observed decrease in malonyl CoA levels produced by metoprolol in control hearts was not attributable to effects on ACC.

**Regulation of Carnitine Palmitoyltransferase-1 and Pyruvate Dehydrogenase**

Because the observed changes in fatty acid oxidation were not explicable on the basis of malonyl CoA levels alone, we investigated whether metoprolol modulates CPT-1 activity and its sensitivity to malonyl CoA. Acute perfusion or chronic treatment with metoprolol both lowered CPT-1 activity (Figure 6A), but the decrease produced by acute perfusion was more marked; chronic metoprolol treatment reduced CPT-1 activity by 32% and 33%, whereas acute metoprolol perfusion decreased CPT-1 activity by 42% and 50%, in control and diabetic hearts respectively. When CPT-1 activity was assayed in the presence of increasing concentrations of malonyl CoA, chronic metoprolol treatment decreased the sensitivity of CPT-1 to malonyl CoA in diabetic, but not control, hearts (Figure 6A). However, acute metoprolol perfusion decreased the sensitivity in both control and diabetic hearts (Figure 6B). Although the maximum CPT-1 activity differed between groups, the rightward shift in the dose-response curve was preserved when the absolute activity data were plotted...
To investigate whether metoprolol could directly inhibit CPT-1 activity or modulate malonyl CoA inhibition, we assayed CPT-1 activity in the presence of a range of concentrations of metoprolol and malonyl CoA (Figure 6C); no inhibition of CPT-1 activity occurred, and malonyl CoA inhibition of CPT-1 was not affected.

To investigate whether metoprolol influences the pyruvate dehydrogenase complex (PDC), we measured PDC activity by in vitro enzyme assay and PDC protein expression by western blotting (Figure 7). Metoprolol decreased PDC catalytic activity without affecting PDC protein expression levels.

Expression of SERCA and ANP

The expression of sarcoplasmic reticulum calcium ATPase (SERCA) was markedly lower in hearts from diabetic animals relative to control hearts, while the expression of atrial natriuretic protein (ANP) was higher in ventricular tissue from diabetic hearts. Chronic metoprolol treatment prevented both of these effects of STZ diabetes (Figure 8).

DISCUSSION

Plasma Parameters and Cardiac Function

Chronic in vivo metoprolol treatment attenuated, but did not completely prevent, the cardiac dysfunction produced by STZ diabetes; the improvement in rate-pressure product being due to an improvement in heart rate rather than in developed pressure. Surprisingly, metoprolol markedly decreased cardiac weight, although the mechanism is not known. It has been shown that chronic
reduction of cardiac load can induce atrophic remodeling by activation of the ubiquitin proteasome proteolytic pathway (38), but it is not clear if metoprolol treatment would produce change in cardiac load to induce such a phenotype in control animals. Further studies are required to determine the mechanism of the decrease in heart weight.

In searching for metabolic correlates to improved function we found that chronic metoprolol treatment did not lead to any significant changes in blood glucose or lipid levels. Surprisingly, however, metoprolol attenuated the increase in ketones seen in the diabetic animals. It is not clear why metoprolol would produce such an effect, but because peripheral ketone utilization appears to depend largely on supply (46), the effect is most likely to reflect a decrease in hepatic ketogenesis. Further work will be required to establish whether metoprolol alters ketogenesis by suppressing lipolysis and therefore the delivery of fatty acids to the liver, by inhibiting fatty acid oxidation in the liver or by some other mechanism.

**Effects of Chronic Metoprolol Treatment on Cardiac Metabolism**

We next measured cardiac substrate utilization *ex vivo* to explore the acute and chronic effects of metoprolol and the mechanisms involved. Chronic metoprolol treatment increased fatty acid oxidation of normal hearts, whereas the higher rates in STZ hearts were reduced. It is well established that inhibition of CPT-1 by malonyl CoA is the major mechanism by which CPT-1 activity is regulated in the heart (19, 25). We therefore hypothesized that metoprolol would inhibit fatty acid oxidation by increasing malonyl CoA levels. Surprisingly, malonyl-CoA concentrations were reduced in control hearts and were unchanged in STZ hearts. To assess CPT-1 more thoroughly, we measured CPT-1 activity in heart tissue. Because allosteric effects on CPT-1 are lost by lysis of the cell membrane and the hydrolysis and dilution of cytosolic metabolites in the assay buffer, this measurement does not
reflect the \textit{in vivo} flux through CPT-1 and is not related to malonyl CoA concentrations in the myocardium. It is, in fact, a measurement of maximum CPT-1 activity. We measured the sensitivity of CPT-1 to malonyl CoA by assaying tissue CPT-1 activity in the presence of increasing concentrations of malonyl CoA. The true flux through CPT-1 \textit{in vivo} is determined by a combination of malonyl CoA levels, CPT-1 maximum activity and CPT-1 sensitivity to malonyl CoA. An indication of the true flux through CPT-1 is given by the measured rates of palmitate oxidation. CPT-1 maximum activity was reduced by metoprolol in both control and diabetic hearts. The sensitivity of CPT-1 to malonyl-CoA inhibition was dramatically reduced by metoprolol in STZ hearts but hardly affected in control hearts.

Therefore, the improvement in heart function of STZ hearts exposed chronically to metoprolol was associated with reduced beta oxidation that is consistent with the reduced total CPT-1 activity. The reduced beta oxidation was not explained by an increase in malonyl-CoA (which was unchanged) nor by a change in CPT-1 sensitivity, which was actually reduced, a change that would tend to enhance beta oxidation. Surprisingly, there seemed to be only modest corresponding increases in glucose oxidation; however, this is partly explained by the fact that metoprolol concurrently inhibited PDC activity. We did not measure lactate oxidation in the present studies, and changes in lactate oxidation are likely to have occurred with metoprolol treatment. However, lactate production, as measured by lactate accumulation in the perfusate, was unaltered by chronic metoprolol treatment.

It is intriguing to note that control and diabetic hearts respond differently to chronic metoprolol treatment. The controls actually show higher \textit{ex vivo} beta oxidation after chronic metoprolol, with a decrease in total CPT-1 and very little change in sensitivity of CPT-1 to malonyl-CoA. In this case, the drop in total tissue malonyl-CoA is the most obvious and likely explanation. The mechanism for the decrease in malonyl CoA levels is not clear. We could find no evidence for changes in the expression of ACC or MCD, nor AMP-activated protein kinase (AMPK) or protein kinase A
(PKA) – mediated phosphorylation of ACC (12). It is possible that malonyl CoA levels fell as a result of decreased availability of acetyl CoA to acetyl CoA carboxylase, occurring as a consequence of CPT-1 inhibition; this speculation would, however, be difficult to test.

Metoprolol decreased active PDC activity without affecting total PDC levels, suggesting that metoprolol increases the inhibition of PDC. These changes would be expected to cause a decrease in glucose oxidation rather than the increase we actually observed, which serves to emphasize the importance of non-covalent mechanisms in the control of glucose oxidation. Furthermore, when insulin was removed from the perfusate, the effects of metoprolol on fatty acid oxidation were preserved despite the fact that the effects on glucose oxidation were lost. These data suggest that metoprolol’s primary action is to inhibit fatty acid oxidation independent of insulin. Metoprolol did not produce direct pharmacological inhibition of CPT-1, indicating that the effect is receptor-mediated. The improvements in glucose oxidation are mediated by the Randle Cycle, and limited by the concurrent direct inhibition of PDC activity.

Effects of Acute Metoprolol Perfusion on Cardiac Metabolism

Rapid effects observed during short-term perfusion of isolated hearts might give important clues about the early events that occur in vivo, likely preceding improvements in function. The higher rates of fatty acid oxidation seen in STZ hearts were reduced acutely by metoprolol. In this case, control hearts responded similarly with inhibition of fatty acid oxidation. Acute metoprolol perfusion also improved myocardial energetics, as measured by tissue adenine nucleotide levels, an improvement which was not seen with chronic treatment. Malonyl CoA levels fell in control hearts and were unchanged in diabetic hearts. CPT-1 activity was reduced, but acute metoprolol perfusion produced a larger decrease than did chronic treatment (acute perfusion: C: 42% reduction, D: 50% reduction;
chronic treatment: C: 32% reduction, D: 33% reduction). Acute metoprolol perfusion decreased the sensitivity of CPT-1 to malonyl CoA in both control and diabetic hearts.

To summarize, metoprolol acutely reduced the high rates of fatty acid oxidation of STZ hearts, with a modest compensation of glucose oxidation. The reduced beta oxidation again seemed to be most influenced by the reduced maximum CPT-1 activity and was not explained by an increase in malonyl-CoA or an increase in sensitivity of CPT-1 to malonyl-CoA (in fact, the reverse was seen). The fact that CPT-1 changes so rapidly, before expression could conceivably change, suggests that a covalent modification of CPT-1 is occurring.

It is not clear whether all β-blockers can inhibit fatty acid oxidation, or even whether the effect is mediated by β-adrenoceptors. Furthermore, because cardiac metabolism is driven by cardiac function (40), some of the effects of metoprolol on cardiac metabolism may be attributable to, rather than responsible for, its effects on cardiac function. Further studies need to assess whether the effect is preserved in cells, in which the effects of cardiac function and the Frank-Starling mechanism do not apply.

Myocardial Remodeling

Surprisingly, metoprolol markedly decreased cardiac weight, although this was not associated with impairment of cardiac function. It is not clear whether the effect was due to apoptosis or atrophy. There are, to our knowledge, no clinical reports of metoprolol inducing either atrophy or apoptosis in the heart. Mechanical unloading of the adult Wistar rat heart has been shown to induce atrophic remodeling by inducing early activation of the ubiquitin proteasome proteolytic pathway (38).
However, it is unclear whether metoprolol treatment would produce sufficient cardiac unloading to induce such a phenotype in control animals. Further studies are required to determine the mechanism of the decrease in heart weight. Intriguingly, metoprolol decreased the heart weight/ body weight ratio in control but not diabetic hearts, indicating that structural and cellular-molecular remodeling are independently affected by metoprolol in diabetes.

STZ diabetes is known to be associated with impairment of calcium handling by the cardiomyocyte which are associated with a marked decrease in SERCA expression and function; restoration of SERCA function ameliorates cardiac dysfunction (11). Consistent with previous measurements in heart failure patients (32), we observed that metoprolol increased SERCA expression in diabetic cardiomyopathy, an important mechanism by which metoprolol could improve cardiac function in this model. It has been reported that chronic inhibition of CPT-1 improves calcium handling and SERCA expression (39, 44) so it is conceivable that the improvement in SERCA expression could be explained on the basis of CPT-1 inhibition. However, SERCA control is likely influenced by multiple factors, not just as a result of CPT-1 effects, as indicated by the parallel effects of metoprolol on ANP expression.

**Conclusions**

Metoprolol treatment ameliorates the decline in function seen in STZ-diabetic rat hearts and this may be explained, at least in part, by a reduction in CPT-1 activity and fatty acid oxidation. In comparison, the allosteric control of CPT-1 by malonyl-CoA appeared not to play a major role in the actions of metoprolol. (b) The rapid effects of metoprolol on CPT-1 activity during ex vivo heart perfusion suggests the importance of acute control through covalent modification. (c) The effects of metoprolol on the expression of SERCA and ANP suggesting that improvements in cardiac function
and metabolism also involve parallel improvements in calcium handling and reversal of fetal gene expression.

**Acknowledgments**

We thank Jerzy Kulpa for HPLC analyzes of CoA ester and adenine nucleotides. We thank Violet Yuen and Mary Battell for their expert technical assistance, and Karen Win, Dale Dhillon, Shahileen Remtulla and Liza Tong for assistance with preliminary experiments. We thank Sherry Wu and Varun Saran for analyzes of ANP levels. We thank Dr. Jason Dyck and Dr. Gary Lopaschuk (University of Alberta, Edmonton, Alberta) for the generous gift of MCD antibodies. We thank Astra-Zeneca and Apotex for the generous gift of metoprolol. This work was supported by the Heart and Stroke Foundation of B.C. and Yukon. V.S. was a recipient of an Rx&D/ CIHR Graduate Research Scholarship in Pharmacy and a Canadian Diabetes Association Doctoral Research Award.

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**Figure 1:** Mechanical performance of Isolated Perfused Hearts. The average heart rate, cardiac output and hydraulic power over the course of the 60-minute perfusion are presented. Data represent means ± SEM and were analyzed using one-way ANOVA with Neumann Keuls post-hoc test. * = significantly different from C, CT and DT, # = significantly different from C and D, + = significantly different from C, CT and D (C=control, n=5; CT=control treated, n=5; D= diabetic, n=5; DT= diabetic treated, n=5).

**Figure 2:** Effects of Chronic In Vivo Metoprolol Treatment on Metabolism of Isolated Perfused Hearts. Glucose and palmitate oxidation, and lactate accumulation in the perfusate during a 60 minute aerobic perfusion. Perfusions were carried out in the presence of insulin (100 µUnits/ml). Data represent means ± SEM. Data were analyzed using One-Way ANOVA with Neumann Keuls post-hoc test, * = significantly different from C of metoprol, + = significantly different from corresponding untreated group p<0.05.

**Figure 3:** Acute Effects of Metoprolol on Metabolism of Isolated Perfused Hearts. Glucose and palmitate oxidation, and lactate accumulation in the perfusate during a 60 minute aerobic perfusion. Perfusions were carried out in the presence of insulin (100 µM/ml). Data represent means ± SEM. Data were analyzed using One-Way ANOVA with Neumann Keuls post-hoc test, * = significantly different from C of metoprol, + = significantly different from corresponding untreated group, p<0.05.

**Figure 4:** A: Expression of ACC and MCD measured by Western blotting. (C=control; CT=control treated; D=diabetic; DT=diabetic treated). Band intensity was quantified using ImageJ software. Data were analyzed using an unpaired Student’s t-test. *=significantly different p<0.05. (C=control; CT=control treated; D=diabetic; DT=diabetic treated).
Figure 5: Phosphorylation of ACC measured by Western blotting. Band intensity was quantified using ImageJ software. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. (C=control; CT=control treated; D=diabetic; DT=diabetic treated).

Figure 6: A: CPT-1 Activity in whole tissue homogenates. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding untreated group, # = significantly different from all other groups (p<0.05) (C=control, n=5; CP = control perfused, n=5 (Conc of metoprolol?); CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5) B. Malonyl CoA IC50 values calculated following curve-fitting analysis of CPT-1 dose-response curves. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from control, + = significantly different from corresponding untreated group, p<0.05. (C=control, n=5; CP = control perfused, n=5; CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5). C. CPT-1 Activity following incubation of control tissue homogenates with increasing concentrations of metoprolol and in the presence of 0, 50 or 100µM malonyl CoA. Data represent means ± SEM (n=5).

Figure 7: A: Active PDC Activity in whole tissue homogenates. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from C, DP and DT, # = significantly different from C, CP, CT, D and DT, + = significantly different from C, CP, CT, D and DP, p<0.05. (C=control, n=5; CP = control perfused, n=5; CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5), B: Expression of PDC
measured by Western blotting using an antibody against the E2 subunit of PDC. Isolated mitochondria were used as a positive control and cytosolic extracts as a negative control. Band intensity was quantified using ImageJ software. Band intensity was normalized to Ponceau stain and expressed as a percentage of control. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. (C=control, n=5; CP = control perfused, n=5; CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5).

**Figure 8:** A: Pro-ANP levels in whole tissue homogenates. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from all groups, p<0.05 (C=control, n=5; CP = control perfused, n=5; CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5). B: Expression of SERCA measured by Western blotting. Band intensity was quantified using ImageJ software. Data were analyzed using an unpaired Student’s t-test. *=significantly different p<0.05. (C=control; CT=control treated; D=diabetic; DT=diabetic treated).
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>486.4 ± 31.3</td>
<td>480.3 ± 41.0</td>
<td>387.8 ± 37.5*</td>
<td>351.6 ± 53.7*</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>1.82 ± 0.10</td>
<td>1.47 ± 0.07*</td>
<td>1.51 ± 0.10*</td>
<td>1.38 ± 0.07+</td>
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<tr>
<td>Heart Weight/ Body Weight Ratio</td>
<td>3.60 ± 0.12</td>
<td>3.18 ± 0.08*</td>
<td>3.85 ± 0.16</td>
<td>3.62 ± 0.11</td>
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<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>7.25 ± 0.26</td>
<td>7.35 ± 0.40</td>
<td>27.99 ± 1.1*</td>
<td>24.09 ± 5.41*</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>1.59 ± 0.41</td>
<td>1.78 ± 0.78</td>
<td>0.49 ± 0.3*</td>
<td>0.37 ± 0.12*</td>
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<tr>
<td>Plasma Triglycerides (mmol/l)</td>
<td>0.19 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.25 ± 0.05*</td>
<td>0.26 ± 0.05*</td>
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<tr>
<td>Plasma Cholesterol (mmol/l)</td>
<td>1.89±0.05</td>
<td>1.86±0.10</td>
<td>2.05±0.18*</td>
<td>2.10 ± 0.15*</td>
</tr>
<tr>
<td>Plasma Free Fatty Acids (mmol/l)</td>
<td>0.19 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Plasma Ketones (mmol/l)</td>
<td>0.76 ± 0.05</td>
<td>0.50 ± 0.04</td>
<td>2.43 ± 0.57*</td>
<td>1.36 ± 0.28+</td>
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</table>

General characteristics and plasma parameters at termination. Animals were fasted for 5 hours prior to blood collection. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from C, + = significantly different from untreated group (p<0.05) (C=control, n=8; CT=control treated with metoprolol, n=8; D=diabetic, n=8; DT=diabetic treated with metoprolol, n=8).
Myocardial energetics and AMPK activity following chronic metoprolol treatment and ex-vivo cardiac perfusion in the presence of insulin. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding unperfused group, + = significantly different from corresponding untreated group (p<0.05) (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (µmol/g wet weight)</td>
<td>6.3 ± 0.3</td>
<td>7.2 ± 1.7</td>
<td>6.0 ± 0.5</td>
<td>5.1 ± 1.2</td>
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<tr>
<td>ADP (µmol/g wet weight)</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>1.6 ± 0.5 +</td>
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<tr>
<td>AMP (µmol/g wet weight)</td>
<td>0.74 ± 0.09</td>
<td>1.03 ± 0.08</td>
<td>0.83 ± 0.08</td>
<td>0.77 ± 0.03</td>
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<tr>
<td>α-1 AMPK Activity (pmol ATP incorporated/ min/ mg protein)</td>
<td>2.7 ± 0.3</td>
<td>3.5 ± 0.6</td>
<td>3.1 ± 0.4</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>α-2 AMPK Activity (pmol ATP incorporated/ min/ mg protein)</td>
<td>3.6 ± 0.4</td>
<td>3.6 ± 0.3</td>
<td>3.4 ± 0.6</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>α-pan AMPK Activity (pmol ATP incorporated/ min/ mg protein)</td>
<td>4.0 ± 0.9</td>
<td>3.8 ± 0.3</td>
<td>3.6 ± 0.5</td>
<td>3.7 ± 0.4</td>
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Table 3

<table>
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<tr>
<th></th>
<th>C</th>
<th>CP</th>
<th>D</th>
<th>DP</th>
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<tr>
<td>ATP (µmol/g wet weight)</td>
<td>6.3 ± 0.3</td>
<td>7.3 ± 0.4 +</td>
<td>6.0 ± 0.5</td>
<td>9.7 ± 0.9 +</td>
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<tr>
<td>ADP (µmol/g wet weight)</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td>3.3 ± 0.4 +</td>
</tr>
<tr>
<td>AMP (µmol/g wet weight)</td>
<td>0.74 ± 0.03</td>
<td>0.22 ± 0.03 +</td>
<td>0.83 ± 0.08</td>
<td>0.39 ± 0.10 +</td>
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<tr>
<td>α-1 AMPK Activity</td>
<td>2.7 ± 0.3</td>
<td>3.8 ± 0.1 +</td>
<td>3.1 ± 0.4</td>
<td>3.7 ± 0.4</td>
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<tr>
<td>α-2 AMPK Activity</td>
<td>3.6 ± 0.4</td>
<td>3.0 ± 0.5</td>
<td>3.4 ± 0.6</td>
<td>3.8 ± 0.7</td>
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<tr>
<td>α-pan AMPK Activity</td>
<td>4.0 ± 0.9</td>
<td>4.0 ± 0.9</td>
<td>3.6 ± 0.5</td>
<td>3.3 ± 0.4</td>
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Myocardial energetics and AMPK activity following ex-vivo cardiac perfusion with metoprolol in the presence of insulin. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. + = significantly different from corresponding untreated group (p<0.05) (C=control, n=5; CP = control perfused with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol).
Table 4

<table>
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<tr>
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<th>CT</th>
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<tr>
<td>Tissue Glycogen Levels</td>
<td>180.4±31.2</td>
<td>160.6±18.1</td>
<td>95.9±18.3</td>
<td>285.0±32.0</td>
<td>237.3±35.7</td>
<td>286.6±35.2</td>
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<td>(µmol/ g dry weight)</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
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<tr>
<td>Tissue Triglyceride</td>
<td>41.1±2.6</td>
<td>35.3±3.0</td>
<td>31.2±1.5</td>
<td>59.1±3.9</td>
<td>31.5±0.8</td>
<td>44.6±2.7</td>
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<td>Levels (µmol/ g dry</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>weight)</td>
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<tr>
<td>Malonyl CoA Levels</td>
<td>17.1±2.4</td>
<td>8.1±1.1</td>
<td>7.7±1.4</td>
<td>20.7±5.2</td>
<td>22.1±4.7</td>
<td>17.4±3.8</td>
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<tr>
<td>(µmol/ g wet weight)</td>
<td></td>
<td></td>
<td>+</td>
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</table>

Tissue glycogen, triglyceride and malonyl CoA levels following chronic treatment with metoprolol and perfusion in the presence of insulin. Data represent means ± SEM. Data were analyzed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding unperfused group, + = significantly different from corresponding untreated group, p<0.05 (C=control, n=5; CP = control perfused, n=5; DT = control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT = control treated, n=5).
Figure 1
TREATMENT GROUP

GLUCOSE OXIDATION
100 µg/ml Insulin Present (nmol/min/g dry weight)

0 100 200 300 400 500 600

CT CT CT CT

TREATMENT GROUP

PALMITATE OXIDATION
1000 µg/ml Insulin Present (nmol/min/g dry weight)

0 200 400 600 800 1000 1200 1400 1600 1800 2000

CT CT CT CT

TREATMENT GROUP

LACTATE PRODUCTION (nmol/min/g dry weight)

0 2 4 6 8 10 12 14 16 18 20

CT CT CT CT

TREATMENT GROUP

Figure 2
Figure 3
Figure 4
Figure 5
A.

![Graphs showing CPT-1 activity levels and treatment groups.](image)

B.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Malonyl CoA (µM)</th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.5 ± 0.17</td>
<td></td>
<td>31.7 ± 8.0</td>
<td>9.0 ± 0.1*</td>
<td>163.5 ± 11.7*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>63.0 ± 6.3*</td>
<td></td>
<td>113.6 ± 22.9*</td>
<td>9.0 ± 0.1*</td>
<td>63.0 ± 6.3*</td>
</tr>
</tbody>
</table>

C.

![Graph showing Metoprolol concentration and CPT-1 activity.](image)

Figure 6
A.

<table>
<thead>
<tr>
<th>Active PDC (mU/ mg protein)</th>
<th>C</th>
<th>CP</th>
<th>CT</th>
<th>D</th>
<th>DP</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.9±0.9</td>
<td>4.7±0.8*</td>
<td>5.8±0.9*</td>
<td>4.9±1.2*</td>
<td>1.0±0.2#</td>
<td>2.8±0.7+</td>
<td></td>
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</table>

B.

Figure 7
A.

![Graph showing pro-ANP levels across different treatment groups (C, CT, D, DT). The y-axis represents pro-ANP levels (nmol/mg protein) and the x-axis represents treatment groups.]

B.

![Bar charts comparing SERCA expression (normalised to total protein) across different treatment groups (C, CT, D, DT).]

Figure 8