β-Agonist stimulation produces changes in cardiac AMPK and coronary lumen LPL only during increased workload


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impaired left ventricular systolic function (cardiomyopathy; see Ref. 69). In vascular smooth muscle, overexpression of LPL brings about FA loading and vascular dysfunction (16).

Heart failure, coupled with conditions such as coronary artery disease and hypertension, is often associated with activation of the sympathetic nervous system and higher levels of circulating catecholamines (14, 17). Although the β-agonist isoproterenol (Iso) had no influence on myocardial FA metabolism, the objective of the present study was to investigate whether β-agonist stimulation influences LPL at its functionally relevant location, the coronary lumen. We demonstrate that Iso does indeed augment luminal LPL, likely secondary to an increase in workload and excessive energy expenditure, rather than a direct effect.

MATERIALS AND METHODS

Experimental animals. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and the University of British Columbia (UBC; Animal Care Certificate no. A04-1009). Adult male Wistar rats (220–240 g) were obtained from the UBC Animal Care Unit and supplied with a standard laboratory diet (PMI Feeds, Richmond, VA) and water ad libitum. All of the experiments were conducted between 0900 and 1300.

In vivo hemodynamics. Rats were anesthetized with 65 mg/kg pentobarbital sodium, and a cannula was inserted in the carotid artery to measure systemic blood pressure and heart rate. Anesthesia is known to cause cardiac depression. After stabilization, animals were either injected intraperitoneally with saline or 10 μg/kg of the β-agonist Iso. Blood pressure and heart rate were recorded every 20 min for 1 h subsequent to Iso.

Isolated working heart perfusion. Hearts were isolated and perfused as described previously (1). Briefly, rats were anesthetized using 2–3% halothane, and the hearts were carefully excised. Rats were not injected with heparin before death because it displaces LPL bound to HSPG on the capillary endothelium. Consequently, it was necessary to cannulate the heart quickly to avoid clotting of blood in the coronary arteries. After cannulation of the aorta, hearts were secured by tying below the innominate artery and perfused retrogradely with Krebs-Henseleit (KH) buffer containing 10 mM glucose (as the only substrate) and 2 mM calcium (pH 7.4, 37°C). Perfusion fluid was continuously gassed with 95% O2-5% CO2. After 10 min, hearts were then switched to a working mode (perfusion through the left atria) using a Direcwin physiograph (Raytech). Where indicated, Iso (10 μM) was then added to the perfusate.

Isolated Langendorff heart perfusion. Hearts were isolated and perfused as described previously (46, 50). Briefly, rats were anesthetized, and the hearts were carefully excised. After cannulation of the aorta, hearts were perfused retrogradely with KH buffer containing 10 mM glucose (as the only substrate). The rate of coronary flow (7–8 ml/min) was controlled by a peristaltic pump. Where indicated, Iso (10 μM) was then added to the KH buffer.

Coronary lumen LPL activity. To measure coronary endothelium-bound LPL, the perfusion solution was changed to buffer containing 1% FA-free BSA and heparin (5 U/ml). This concentration of heparin can maximally release cardiac LPL from its HSPG binding sites (44, 50). The coronary effluent (perfusate that drips down to the apex of the heart) was collected in timed fractions (10 s) over 5 or 10 min where indicated and assayed for LPL activity by measuring the hydrolysis of a sonicated [3H]triolein substrate emulsion (52). Retrograde perfusion of whole hearts with heparin results in a discharge of LPL that is rapid (within 0.5–1 min; suggested to represent LPL located at or near the endothelial cell surface) followed by a prolonged slow release (considered to represent enzyme that originates from the myocyte cell surface). Because we were primarily concerned with examining regulation of LPL at the coronary lumen, only peak LPL activities are shown. LPL activity is expressed as nanomoles oleate released per hour per milliliter. Subsequent to LPL displacement with heparin, hearts were rapidly removed, washed with Krebs buffer, frozen in liquid nitrogen, and stored at −80°C for Western Blot assay of AMPK and ACC.

Isolated cardiomyocytes. Ventricular calcium-tolerant myocytes were prepared by a previously described procedure (4–50). Briefly, myocytes were made calcium-tolerant by successive exposure to increasing concentrations of calcium. Our method of isolation yields a highly enriched population of calcium-tolerant myocardial cells that are rod-shaped in the presence of 1 mM Ca2+, with clear cross striations. Intact cells are intact but hypercontract into vesiculated spheres. Yield of myocytes was determined microscopically using an improved Neubauer hemocytometer. Myocyte viability was assessed as the percentage of elongated cells with clear cross striations that excluded 0.2% trypan blue. Cardiac myocytes were suspended at a final cell density of 0.4 × 106 cells/ml, incubated with or without Iso (10 μM) at 37°C for 1 h, and basal LPL activity in the medium and cell pellet (after centrifugation) was measured. To release surface-bound LPL activity, heparin (5 U/ml) was then added to the myocyte suspension. After incubation for 10 min, an aliquot of cell suspension was removed, and medium was separated by centrifugation in an Eppendorf microcentrifuge (1 min, 10,000 g) and assayed for LPL activity. The total cellular LPL activity was measured by sonication (Vibra Cell sonicator at a frequency of 40 Hz for 2 × 30 s) of the cell pellets after resuspending them in 0.2 ml of 50 mM NH4Cl buffer (pH 8.0) containing 0.125% (vol/vol) Triton X-100. After sonication, the volume was adjusted to 1 ml using a sucrose buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM HEPES, pH 7.4). Assay for cell sonicate LPL activity was done using 20 μl of the cell sonicate and heparin (2 U/ml).

Cardiac LPL gene expression. LPL gene expression was measured in the indicated groups using RT-PCR (2, 44). Briefly, total RNA from hearts (100 mg) was extracted using TRIzol (Invitrogen). After spectrophotometric quantification and resolving of RNA integrity on a 1% agarose gel containing ethidium bromide. Expression levels were set at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, for a total of 30 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide. Expression levels were represented as the ratio of signal intensity for LPL mRNA relative to β-actin mRNA.

Western Blotting for AMPK and ACC. AMPK phosphorylation increases its activity ~50- to 100-fold (53, 54, 58). Activated AMPK phosphorylates and inactivates ACC (53, 54, 58). To determine total and phosphorylated AMPKαs and ACC, whole cell homogenates were isolated as described previously (3). Briefly, hearts were ground under liquid nitrogen, and 50 mg were homogenized. After centrifugation at 5,000 g for 20 min, the protein content of the supernatant was
quantified using a Bradford protein assay. Samples were diluted and boiled with sample loading dye, and 50 μg were used in SDS-PAGE. After transfer, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated either with rabbit AMPKα, phospho-AMPK (Thr172), or phospho-ACC (Ser79) antibodies and subsequently with secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies and visualized using enhanced chemiluminescence (ECL) detection. Measuring the phospho form of AMPK is a surrogate for estimation of its activity.

Separation and characterization of cardiac lipids. Total cardiac lipids were extracted and solubilized in chloroform-methanol-acetone-hexane (4:6:1:1 vol/vol/vol/vol). Separation of TG and FA was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA) equipped with an auto-sampler and column heater. FA were quantified as their respective methyl esters using heptadecaenoic acid (17:0) as the internal standard with a Varian 3400 GLC equipped with a flame ionization detector, a Varian Star data system, and a SP-2330 capillary column (30 m × 0.25 mm; Supelco, Bellefonte, PA). Value of cardiac FA and TG were expressed as micrograms per milligram protein.

Serum measurements. Blood samples were removed from animals and centrifuged immediately to collect serum, which was then stored at −20°C until assay. Diagnostic kits were used to measure glucose, TG (Sigma), and nonesterified FA (Wako).

Statistical analysis. Wherever appropriate, one-way ANOVA followed by the Tukey or Bonferroni tests or the unpaired and paired Student’s t-test were used to determine differences between group mean values (as indicated in the specific legends for Figs. 1–6). The level of statistical significance was set at \( P < 0.05 \).

Materials. Iso was purchased from Sigma. Total AMPKα, phospho-AMPKα, and phospho-ACC antibodies were obtained from Cell Signaling (Beverly, MA). The ECL detection kit was obtained from Amersham.

RESULTS

Coronary luminal LPL activity increases after a single dose of Iso given in vivo. After Iso administration, RPP increased significantly compared with control (Table 1). Changes in plasma parameters after 1 h of Iso included a significant increase in serum-free FA, with no effect on either glucose or TG (Table 1). To determine whether Iso controls LPL (at its functionally relevant location, the coronary lumen), hearts isolated from Iso-treated animals were perfused retrogradely with heparin. Compared with saline-injected rats, there was a substantial increase in luminal LPL activity (∼300%) at the vascular lumen after 1 h of Iso (Fig. 1). This increase in LPL activity was maintained for an additional 3 h (Fig. 1).

Table 1. General characteristics of the animals

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<thead>
<tr>
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<th>Control</th>
<th>Iso</th>
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<tr>
<td>Blood glucose, mM</td>
<td>5.7±0.2</td>
<td>5.4±0.2</td>
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<tr>
<td>Serum free fatty acid, mM</td>
<td>0.34±0.04</td>
<td>0.49±0.05*</td>
</tr>
<tr>
<td>Serum triglyceride, mM</td>
<td>1.4±0.2</td>
<td>1.3±0.1</td>
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<tr>
<td>Systolic pressure, mmHg</td>
<td>116±4</td>
<td>131±10</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>421±16</td>
<td>474±18*</td>
</tr>
<tr>
<td>Rate-pressure product</td>
<td>49,192±3,121</td>
<td>62,093±5,378*</td>
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Values are means ± SE for 6 animals in each group. Rats were anesthetized with 25 mg/kg pentobarbital sodium, and a cannula was inserted in the carotid artery to measure systemic blood pressure and heart rate. After stabilization, animals were either injected ip with saline or 10 μg/kg of the β-agonist isoproterenol (Iso). Blood pressure and heart rate were recorded every 20 min for 1 h, and averages are presented. For measurement of serum parameters, blood was collected at termination (1 h after Iso). *Significantly different from control, \( P < 0.05 \).

Notably, alterations in luminal LPL activity were independent of shifts in mRNA levels (Fig. 1, bottom). Iso injected intraperitoneally augments cardiac AMPK and ACC phosphorylation. Because changes in luminal LPL activity were independent of shifts in LPL mRNA, the LPL increase at the coronary lumen is likely via posttranscriptional mechanisms. Interestingly, upon injection of Iso, the increase in LPL was also associated with phosphorylation of AMPK, both at 1 and 4 h after administration (Fig. 1, bottom). Once activated, AMPK phosphorylates and inactivates ACC. As ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, AMPK, by phosphorylating ACC, is able to decrease malonyl-CoA and minimize its inhibition of FA oxidation (53, 54, 58). In the heart, ACC280 (280-kDa) is the predominant isoform. After 1 h of Iso, phosphorylation of ACC280 increased; although ACC280 phosphorylation appeared higher at 4 h, no statistical difference could be identified (\( P = 0.0896 \); Fig. 2, bottom). Interestingly, Iso induced a time-dependent drop in cardiac TG, without affecting cardiac free fatty acid (FFA; Table 2).

Iso does not influence LPL activity and AMPK phosphorylation in myocytes or Langendorff-perfused hearts. Previous studies have shown that incubation of cardiomyocytes with Iso for 30 min had no effect on either basal or HR-LPL activity (60). In the present study, incubation with this β-agonist was...
extend to 1 h, and no effect was observed on basal (unpublished observation), HR (control, 847 ± 0.02 Hz; Iso, 814 ± 155 nmol/h/10^6 cells), or cell sonicate (control, 2,287 ± 317; Iso, 2,142 ± 354; nmol/h/10^6 cells) LPL activity. To determine whether Iso influences LPL in the intact heart, we perfused hearts retrogradely with this β-agonist. Retrograde perfused hearts allow for adequate coronary perfusion with limited expenditure of energy (these hearts do not beat against an afterload). Similar to myocytes, Iso did not change HR-LPL in Langendorff isolated hearts (Fig. 3). Overall, our data suggest that, specifically in these preparations in vitro, Iso does not have a direct effect in regulating LPL activity. In a recent report, we described that recruitment of LPL to the coronary lumen can be regulated by AMPK (2). The relationship between AMPK phosphorylation and LPL activity was evaluated in isolated myocytes and Langendorff-perfused hearts. We demonstrate that Iso did not affect AMPK phosphorylation in either myocytes and Langendorff-perfused hearts. We demonstrate that Iso did not affect AMPK phosphorylation in either myocytes (as measured by densitometry; control, 0.97 ± 0.02; Iso, 0.88 ± 0.04 units) or retrograde perfused hearts (Fig. 3; as measured by densitometry; control, 1.0 ± 0.1; Iso, 1.03 ± 0.14 units).

Increasing workload promotes phosphorylation of AMPK and ACC and enlarges the coronary lumen LPL pool. Given that the Iso-induced increase in RPP was associated with phosphorylation of AMPK and ACC, and coronary luminal LPL activity in vivo, and that myocytes or retrograde perfused hearts do not represent a physiological contractile circumstance, we considered whether simply increasing workload by switching from Langendorff to the isolated perfused working heart would induce similar changes. Compared with retrograde perfusion, the working heart perfused only with glucose (but in the absence of insulin) demonstrated higher phospho-AMPK (Fig. 4; as measured by densitometry; Langendorff heart, 0.91 ± 0.15; working heart, 2.15 ± 0.6 units, P < 0.05), and phospho-ACC280 (Fig. 4; as measured by densitometry; Langendorff heart, 0.9 ± 0.08; working heart, 1.4 ± 0.04 units, P < 0.05), and HR-LPL activity (Fig. 4). These results suggest that, when increasing workload augments cardiac mechanical performance and energy expenditure, LPL is recruited to the coronary lumen.

**Fig. 2.** AMP-activated protein kinase (AMPK) and 280-kDa acetyl-CoA carboxylase (ACC280) phosphorylation in hearts isolated from animals injected with Iso for 1 or 4 h. After Iso injection for 1 or 4 h, hearts were removed and snap-frozen in liquid nitrogen. AMPKα (total and phosphorylated; top) and phospho-ACC280 (bottom) were measured using Western blotting and rabbit AMPKα, phospho-AMPK (Thr172), or phospho-ACC280 (Ser79) antibodies. Data are means ± SE of 5 different hearts in each group. *Significantly different from control, P < 0.05.

**Table 2.** Cardiac lipids in control and Iso-treated groups

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<th>Triglyceride, µg/mg protein</th>
<th>Free Fatty Acid, µg/mg protein</th>
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<tr>
<td>Control</td>
<td>1.7 ± 0.2</td>
<td>4.6 ± 0.5</td>
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<tr>
<td>Iso 1 h</td>
<td>1.1 ± 0.1*</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Iso 4 h</td>
<td>0.7 ± 0.1*</td>
<td>3.4 ± 0.1</td>
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Values are means ± SE for 4 animals in each group. After in vivo injection of Iso (1 or 4 h) hearts were collected, and cardiac triglyceride and free fatty acid were measured using HPLC. *Significantly different from control, P < 0.05.
Augmentation of substrate supply reduces AMPK and ACC phosphorylation and HR-LPL activity in the isolated perfused working heart.

Glucose entry in the heart is predominantly insulin dependent (7). Compared with glucose, FA is the preferred substrate and, when supplied at physiological levels, contributes ~70% of the ATP necessary for normal heart function (47, 48). Interestingly, provision of insulin and albumin-bound palmitic acid to the working heart reduced phosphorylation of AMPK (Fig. 5, top), ACC280 (Fig. 5, bottom), and HR-LPL activity (Fig. 6) without changing the RPP (glucose only, 25,400 ± 2,000; insulin/palmitate, 24,700 ± 300). In these hearts, introduction of Iso to the buffer perfusate increased RPP (control, 24,700 ± 300; Iso, 33,600 ± 900, P < 0.05) and reversed the effects on AMPK and ACC280 phosphorylation and LPL activity (Figs. 5 and 6). Taken together, our results suggest that the Iso-induced increase in AMPK and ACC phosphorylation occurs through indirect mechanisms.

**DISCUSSION**

FA delivery and utilization by the heart involves 1) release from adipose tissue and transport to the heart after complexing with albumin (35), 2) provision through the breakdown of endogenous cardiac TG stores (42, 57), 3) internalization of whole lipoproteins (20, 61, 64), and 4) hydrolysis of circulating TG-rich lipoproteins to FA by LPL positioned at the endothelial surface of the coronary lumen (5). It should be noted that the molar concentration of FA bound to albumin is ~10-fold less than that of FA in lipoprotein-TG (39), and, recently, LPL-mediated hydrolysis of lipoproteins to FA was suggested to be the principal source of FA for cardiac utilization (4). LPL is synthesized in cardiomyocytes and subsequently secreted onto HSPG binding sites on endothelial cells in the coronary lumen (13, 15, 39, 41). Thus, even though the majority of enzyme is located in myocytes, vascular endothelial-bound LPL likely determines the rate of plasma lipoprotein-TG clearance (41). Previously, incubation of cardiomyocytes with Iso for 30 min did not change either HR-LPL or cellular LPL activity (60). We have confirmed these findings even after incubation with Iso was prolonged for 1 h. Because cardiomyocytes are quiescent, we extended these experiments to an isolated retrograde perfused heart and demonstrated a similar absence of effect of this β-agonist on LPL. The models above are not truly representative of a heart beating in vivo. In the only study that examined the influence of Iso in the intact animal, LPL activity was measured in whole heart homogenates (12), which does not distinguish between the HR (localized on capillary endothelial cells) and cellular (that represents a storage form of the enzyme) pools of LPL. Our results, for
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In summary, our data suggest that Iso can influence HR-LPL only during conditions of increased workload and excessive energy expenditure and is highly associated with phosphorylation of AMPK. Whether this increase in LPL after Iso can induce cardiovascular damage is currently unknown. However, LPL-mediated lipoprotein hydrolysis can provide excessive FA that may affect the biology of the vessel wall (9, 16). Indeed, when endothelial cell monolayers are incubated with increasing concentrations of FA, the permeability of the monolayers is enhanced, allowing albumin to traverse faster across the vessel wall (24). Additionally, tissue-specific overexpression of LPL is associated with severe myopathy (27, 34, 67) and promotion...
of atherosclerosis (16). Thus a novel mechanism by which excessive β-agonists could induce cardiovascular complications is through their control of HR-LPL.

GRANTS

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REFERENCES


