Ketone bodies alter dinitrophenol-induced glucose uptake through AMPK inhibition and oxidative stress generation in adult cardiomyocytes

Amélie Pelletier and Lise Coderre
Montreal Diabetes Research Centre, Centre hospitalier de l’Université de Montréal (CHUM) and the Department of Medicine, Université de Montréal, Montreal, Quebec, Canada

Submitted 18 April 2006; accepted in final form 8 January 2007

Pelletier A, Coderre L. Ketone bodies alter dinitrophenol-induced glucose uptake through AMPK inhibition and oxidative stress generation in adult cardiomyocytes. Am J Physiol Endocrinol Metab 292: E1325–E1332, 2007. First published January 16, 2007; doi:10.1152/ajpendo.00186.2006.—In aerobic conditions, the heart preferentially oxidizes fatty acids. However, during metabolic stress, glucose becomes the major energy source, and enhanced glucose uptake has a protective effect on heart function and cardiomyocyte survival. Thus abnormal regulation of glucose uptake may contribute to the development of cardiac disease in diabetics. Ketone bodies are often elevated in poorly controlled diabetics and are associated with increased cellular oxidative stress. Thus we sought to determine the effect of the ketone body β-hydroxybutyrate (OHB) on cardiac glucose uptake during metabolic stress. We used 2,4-dinitrophenol (DNP), an uncoupler of the mitochondrial oxidative chain, to mimic hypoxia in cardiomyocytes. Our data demonstrated that chronic exposure to OHB provoked a concentration-dependent decrease of DNP action, resulting in 56% inhibition of DNP-mediated glucose uptake at 5 mM OHB. This was paralleled by a diminution of DNP-mediated AMP-activated protein kinase (AMPK) and p38 MAPK phosphorylation. Chronic exposure to OHB also increased reactive oxygen species (ROS) production by 1.9-fold compared with control cells. To further understand the role of ROS in OHB action, cardiomyocytes were incubated with H2O2. Our results demonstrated that this treatment diminished DNP-induced glucose uptake without altering activation of the AMPK/p38 MAPK signaling pathway. Incubation with the antioxidant N-acetylcysteine partially restored DNP-mediated glucose uptake but not p38 MAPK phosphorylation. In conclusion, these results suggest that ketone bodies, through inhibition of the AMPK/p38 MAPK signaling pathway and ROS overproduction, regulate DNP action and thus cardiac glucose uptake. Altered glucose uptake in hyperketonemic states during metabolic stress may contribute to diabetic cardiomyopathy.

glucose transport; hypoxia; 2,4-dinitrophenol; adenosine monophosphate-activated protein kinase

CARDIOVASCULAR DISEASE is a well-known complication of diabetes. Diabetes increases the risk of mortality from heart disease by 4.0- to 6.6-fold and 6.2- to 17.1-fold in men and women, respectively (26). It also significantly worsens the prognosis after heart failure, despite the fact that infarct size is often smaller in diabetics (47). Poorly controlled type 1 diabetic patients often have elevated concentrations of ketone bodies that can reach levels as high as 10 mM during severe ketosis (27). Increased ketone body levels have also been observed in type 2 diabetes (3) and in congestive heart failure (31). Furthermore, in the latter condition, increased plasma ketone body concentrations correlate with the severity of cardiac dysfunction (31).

More than 64% of type 1 and 34% of type 2 diabetic patients have inadequate cardiac glucose uptake (34, 51), and it has been suggested that alteration of this process contributes to the development of diabetic myocardial disease (33). Glucose is the primary energy source for the heart during metabolic stresses such as ischemia or hypoxia (11). Studies have shown that enhanced glucose uptake, by provision of an energy source for ATP synthesis, has a beneficial effect on contractile function and coronary flow during ischemia (11). Conversely, failure to upregulate glucose uptake and to maintain ATP levels during hypoxia is associated with increased cardiomyocyte death (1, 32). Diminished glucose utilization and contractile dysfunction have been reported in the heart of db/db mice, a model of type 2 diabetes (4). These abnormalities were corrected by selective overexpression of GLUT4 in the heart, the major glucose transporter expressed in this tissue (4).

Recently, the signaling cascade involved in the stimulation of glucose uptake by metabolic stress has started to be unraveled and the predominant role of 5′-AMP-activated protein kinase (AMPK) in this process highlighted. AMPK, a sensor of fuel and energy status, is activated in response to an increase in the ratios of AMP to ATP or creatine to phosphocreatine (14, 17) or by phosphorylation of the threonine residue by AMPK kinase (46). AMPK is stimulated in response to the adenosine analog 5-aminomidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) (6, 41), ischemia (42), hypoxia (17, 35), and 2,4-dinitrophenol (DNP) (17, 37). Overexpression of constitutively active AMPK or stimulation with AICAR enhances glucose uptake in skeletal muscle (15) and heart (29, 41). Conversely, overexpression of a dominant-negative (DN) AMPK mutant (52) or a kinase-dead (KD) AMPKα2 isoform (29) partially blocks ischemia- and hypoxia-mediated glucose uptake in the heart. Using a DN-AMPK mutant, we recently demonstrated that AMPK activation is essential for maximal stimulation of glucose uptake in response to DNP in cardiomyocytes (37). Thus AMPK plays a central role in the regulation of glucose uptake during metabolic stress.

p38 MAPK, a member of the MAPK family of serine/threonine protein kinases, is also activated in response to DNP (48), hypoxia (24), and ischemia (5). Recent studies in skeletal muscle (28), cardiomyocytes (37), and the heart (29) suggest that p38 MAPK is a downstream effector of AMPK and may participate in regulation of the glucose uptake process.

Address for reprint requests and other correspondence: L. Coderre, Research Centre, CHUM-Hôtel-Dieu, 3850 rue Saint-Urbain, Montréal, Québec, Canada H2W 1T7 (e-mail: lise.coderre@umontreal.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Studies have reported that hyperketonemic type 1 diabetic patients present increased plasma lipid peroxidation and low levels of cellular glutathione compared with normoketonic patients (20). These results suggest that high levels of ketone bodies increase cellular oxidative stress, which may contribute to the development of cardiac insulin resistance in diabetes (10). We have demonstrated that prolonged exposure to β-hydroxybutyrate (OHB), the main ketone body produced during hyperketonemia (25), induces insulin resistance in cardiomyocytes (49). We were, therefore, interested to determine whether ketone bodies also alter metabolic stress-stimulated glucose uptake and whether increased oxidative stress plays a role in OHB action in cardiomyocytes. We used DNP, a weak base that dissipates the H⁺ gradient and uncouples the mitochondrial oxidative chain, as a chemical model to mimic hypoxia in these cells. Our results demonstrated that chronic exposure to OHB diminished DNP-mediated glucose uptake and that both inhibition of the AMPK/p38 MAPK signaling pathway and reactive oxygen species (ROS) overproduction probably contributed to this effect.

EXPERIMENTAL PROCEDURES

Chemicals. All cell culture solutions, fatty acid-free bovine serum albumin (FABSA), water, supplements, DNP, OHB, N-acetyl-L-cysteine (NAC), hydrogen peroxide (H₂O₂), lucigenin, trypsin, trypsin inhibitor, and DNašeI were purchased from Sigma-Aldrich (Oakville, ON, Canada). Collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Human insulin (Humulin R) was procured from Eli Lilly (Toronto, ON, Canada). Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-AMPK (Thr172), and AMPK polyclonal antibodies were from Cell Signaling Technology (Beverly, MA), while phospho-acetyl-CoA carboxylase (Ser79) (ACC) was from Upstate Cell Signaling Solutions (Lake Placid, NY). DuPont NEN Research Products (Boston, MA) supplied 2-[3H]deoxyglucose (DG). Polyvinylidene difluoride membranes were purchased from Immobilon Millipore (Bedford, MA). The enhanced chemiluminescence detection system was bought from Amersham Pharmacia Biotech (Baie d’Urfe, QC, Canada). The Bradford protein assay kit was from Bio-Rad (Hercules, CA). All electrophoresis reagents were obtained from Boehringer Mannheim (Laval, QC, Canada).

Isolation of adult rat cardiomyocytes. All experiments conformed to guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of CHUM. Male Sprague-Dawley rats weighing 175–200 g were injected (ip) with 500 U of heparin sulfate 15 min before anesthesia with pentobarbital sodium (60 mg/kg). The hearts were excised, and calcium-tolerant cardiomyocytes were isolated by the Langendorff method as described previously (49). During the entire procedure, the cells were maintained at 37°C. Briefly, all hearts were rinsed (4 ml/min) for 5 min in Krebs-1% oxygen (49). During the entire procedure, the cells were maintained at 37°C. They were then perfused with a calcium-free KR solution for 5 min to stop spontaneous cardiac contractions. This was followed by perfusion with KR buffer supplemented with 0.05% collagenase, 15 mM 2,3-butanediol monoxime, and 0.1% FABSA for 15 min. For the last 5 min of perfusion, the KR buffer was supplemented with 0.05 mM CaCl₂, after which the ventricles were separated from the atria. The ventricles were minced in KR supplemented with 0.05% collagenase, 15 mM 2,3-butanediol monoxime, 0.2 mg/ml DNašeI, 0.1 mM CaCl₂, and 0.1% FABSA. The resulting cell suspension was filtered through a nylon mesh and centrifuged at 1,000 g for 45 s. The cells were washed twice and diluted in medium 199 supplemented with 11 mM glucose, 0.2% FABSA, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 10⁻⁷ M insulin, 0.1 M ascorbic acid, 100 IU/ml penicillin, 25 µg/ml gentamicin, and 100 mg/ml streptomycin and then plated onto laminin-coated dishes. Cell viability was determined by the percentage of rod-shaped cells and averaged 90–95%. After 4 h, they were washed to remove damaged cells and debris. The remaining cells were immediately utilized for experiments and were incubated at 37°C for 16 h in media containing 5 mM glucose and 10⁻¹¹ M insulin.

Cardiomyocyte treatments. The cells were incubated with increasing concentrations of OHB (0–5 mM) for 16 h or with 5 mM OHB for 1, 4, or 16 h. In certain experiments, cardiomyocytes were treated with 5 mM OHB alone or in combination with 75 µM NAC for 16 h. To mimic the effect of ROS, cardiomyocytes were also incubated with increasing concentrations of H₂O₂ (0–25 µM) for 16 h. Glucose uptake, ROS production, and enzyme activation were assessed after these treatments.

Glucose uptake in primary cultures of cardiomyocytes. On the day of the study, the cells were washed twice with 1 ml of KR buffer to remove insulin, OHB, NAC, and H₂O₂. They were then incubated for 30 min in 1 ml of KR buffer containing 5 mM glucose and 0.2% FABSA but without insulin, OHB, NAC, or H₂O₂. Thus we evaluated the effect of prior OHB exposure on this process. Glucose uptake assay was started by addition of the vehicle (basal) or 0.1 mM DNP and 1 µCi/ml DG, as described previously (49). After 20 min, glucose uptake measurement was terminated by three rapid washes with 1 ml of ice-cold KR buffer. The cells were disrupted with 0.5 ml of 0.5 M NaOH for 60 min at 37°C, and cell-associated radioactivity was quantified by scintillation counting. Glucose uptake was normalized to total protein, as measured by Bio-Rad assay.

Measurement of ROS production. ROS production was quantified by the lucigenin method (8, 18) with the following modifications. Cardiomyocytes were treated as described for the uptake studies. The cells were washed twice with medium containing 10⁻¹¹ M insulin and 5.5 mM glucose to remove OHB. They were then incubated for 30 min with medium containing 1 mg/ml trypsin, after which trypsin inhibitor (2 mg/ml) was added and the cells collected. The cardiomyocytes were then centrifuged at 1,000 g for 45 s, and the pellet was washed in modified Hanks’ buffer containing (in mM) NaCl (137), KCl (5), MgSO₄ (0.6), CaCl₂ (1.3), Na₂HPO₄ (0.3), KH₂PO₄ (0.4), glucose (5.5), and NaHCO₃ (4.2), pH 7.4. The cells were resuspended in 1 ml of Hanks’ buffer. To measure ROS production, 900 µl of cell suspension were transferred to glass tubes and assessed in a luminometer. We used Hanks’ buffer as a blank. Measurement was started by the addition of 100 µl of lucigenin (0.5 mM, final concentration). Photon emission was counted every 2.5 min for the first 10 min, after which photomission was evaluated every 5 min for an additional 15 min.

AMPK, ACC, and p38 MAPK activation. The cells were washed twice with media containing no insulin, OHB, NAC, or H₂O₂ and incubated for 1 h before stimulation with 0.1 mM DNP for 5 or 10 min. Therefore, the effect of ketone bodies on DNP signaling was evaluated without OHB present in the media. The reaction was stopped by three rapid washes with ice-cold KR buffer. The cells were then lysed in buffer containing 25 mM Tris·HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS). The lysate was centrifuged for 5 min at 12,000 g at 4°C to remove insoluble material, and the resulting supernatant was used for immunoblotting.

Gel electrophoresis and immunoblotting. Samples were electrophoresed on 6 or 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes for Western blotting. The membranes were blocked for 1 h with 5% milk (wt/vol) in PBST, pH 7.4, containing 137 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 0.05% Tween 20, and 0.02% sodium azide. They were then incubated with the primary antibody, followed by incubation with the
appropriate secondary antibody conjugated to horseradish peroxidase. Antigen-antibody complexes were detected by the enhanced chemiluminescence method. Quantitative analysis was performed with a scanning densitometer.

Statistical analysis. Two-way analysis of variance (ANOVA) was applied for multiple comparisons, followed by the Tukey-Kramer post hoc test. Two-tailed Student’s t-test was performed when two groups were compared. All data were reported as means ± SE. P < 0.05 values were considered to be significant.

RESULTS

OHB inhibits DNP-stimulated glucose uptake in a time- and concentration-dependent manner. Poorly controlled diabetic patients are susceptible to severe ketosis. We have demonstrated previously that hyperketonemia induces insulin resistance in cardiomyocytes (49). Because insulin and DNP stimulate cardiac glucose uptake through the activation of independent signaling pathways (37), we were interested in determining whether ketone bodies also altered DNP action in these cells. To address this question, cardiomyocytes were incubated with 5 mM OHB, a concentration known to inhibit insulin-stimulated glucose uptake (49), for 1, 4, or 16 h before evaluation of DNP-stimulated glucose uptake. As illustrated in Fig. 1, stimulation with 0.1 mM DNP induced a 2.3-fold increase of glucose uptake (P < 0.01) in control cardiomyocytes. Preincubation with OHB for 1 or 4 h did not alter DNP action, and 2.6- and 2.9-fold increases in glucose uptake were observed in these cells (P < 0.01), respectively. However, and similar to its effect on insulin action, chronic exposure to OHB (16 h) significantly reduced the maximal stimulation of glucose uptake in response to DNP by 45% (P < 0.05). Since chronic exposure to OHB does not alter GLUT1 and GLUT4 protein concentrations (49), the inhibition of glucose uptake cannot be explained by such changes. All subsequent experiments were performed after 16 h of exposure to OHB.

Then, we established the concentration of OHB required to inhibit DNP action in cardiomyocytes (Fig. 2). Exposure to 0.1 mM OHB for 16 h did not alter DNP action significantly, and a threefold increase in DNP-mediated glucose uptake was observed in these cells (P < 0.01). In contrast, treatment with 0.2, 0.5, or 5 mM OHB significantly reduced DNP action in these cells (P < 0.05, P < 0.05, and P < 0.01, respectively). We also calculated the net DNP stimulatory effect over basal glucose uptake in these cells. OHB inhibited net DNP-stimulated glucose uptake in a concentration-dependent manner, resulting in 26, 45, 45, and 56% reductions of glucose uptake at 0.1, 0.2, 0.5, and 5 mM OHB, respectively (P < 0.05) (data not shown). Because maximal inhibition of this process occurred at 5 mM OHB, all subsequent experiments were performed at this concentration.

OHB reduces DNP-stimulated AMPK, ACC, and p38 MAPK phosphorylation. To understand the molecular mechanisms underling ketone body action, we investigated the effect of chronic exposure to OHB on DNP-mediated activation of the AMPK/p38 MAPK signaling pathway. OHB did not significantly modify the expression of AMPK and p38 MAPK in these cells. AMPK and p38 MAPK activities were examined with antibodies that recognize the phosphorylated and active form of these enzymes. As shown in Fig. 3A, DNP induced a 1.7-fold increase of AMPK phosphorylation (P < 0.01) that was reduced by 45% on chronic exposure to OHB (P < 0.01). We then evaluated ACC phosphorylation, a well-known downstream target of AMPK. Studies have shown that ACC phosphorylation reflects AMPK activation both by allosteric effectors and by kinases. In control cells, ACC phosphorylation was enhanced by 3.6-fold in response to DNP (P < 0.05) (Fig. 3B), and this activation was completely blocked in OHB-treated cells (P < 0.01). We next evaluated p38 MAPK phosphorylation in response to DNP. DNP increased p38 MAPK phosphorylation by 17-fold in control cells (P < 0.01); this increase was completely inhibited by OHB (P < 0.01) (Fig. 3C).

OHB increases ROS production in cardiomyocytes. Studies by Jain et al. (20) have demonstrated that lipid peroxidation is increased in hyperketonemic type 1 diabetic patients compared with normoketoneinic subjects. We therefore examined whether ROS production plays a role in ketone body action. ROS production was determined over 25 min after the addition of lucigenin (Fig. 4A), after which the area under the curve was determined. As shown in Fig. 4B, chronic exposure to OHB increased ROS production by 1.9-fold compared with control cells (P < 0.01). As demonstrated for neuronal cells (22), stimulation with 0.1 mM DNP induced a 50% decrease in ROS...
production both in control and in OHB-treated cardiomyocytes (Fig. 4B). However, this decrease was significant only in ketone body-treated cells ($P < 0.05$).

H$_2$O$_2$ inhibits DNP-stimulated glucose uptake in cardiomyocytes in a concentration-dependent manner. We then determined whether ROS overproduction participates in the inhibitory effect of OHB on DNP-stimulated glucose uptake. Thus cardiomyocytes were incubated with increasing concentrations (5, 10, and 25 μM) of H$_2$O$_2$ for 16 h (Fig. 5). Higher H$_2$O$_2$ concentrations were toxic to the cells (data not shown). Exposure to 5 μM H$_2$O$_2$ did not alter DNP action compared with control cells. A 2.6-fold increase of glucose uptake in response to DNP was measured in these cells ($P < 0.05$). On the other hand, incubation with 10 or 25 μM H$_2$O$_2$ reduced DNP-stimulated glucose uptake by 11 and 29%, respectively. We then calculated the net stimulatory effect of DNP on glucose uptake. Chronic exposure to increasing concentrations of H$_2$O$_2$
provoked a concentration-dependent decrease in net DNP-stimulated glucose uptake ($P < 0.05$) (data not shown). These changes in glucose uptake could not be explained by alterations in GLUT1 and GLUT4 protein content, as both control and H$_2$O$_2$-treated cells expressed similar levels of these proteins (data not shown). Because maximal inhibition of DNP action was obtained with 25 $\mu$M H$_2$O$_2$, subsequent experiments were done with this concentration.

H$_2$O$_2$ does not alter DNP-stimulated AMPK and p38 MAPK phosphorylation. To establish the mechanism by which H$_2$O$_2$ inhibits DNP-stimulated uptake, we evaluated its effect on activation of the AMPK-dependent signaling pathway. H$_2$O$_2$ did not alter AMPK and p38 MAPK protein expression in cardiomyocytes. As shown in Fig. 6A, chronic exposure to H$_2$O$_2$ did not alter AMPK phosphorylation in response to DNP, and a 2.5-fold increase of AMPK phosphorylation was observed in response to DNP in H$_2$O$_2$-treated cells ($P < 0.05$) (Fig. 6B).

NAC improves DNP-stimulated glucose uptake in OHB-treated cardiomyocytes. Because oxidative stress alters DNP-stimulated glucose uptake, we investigated whether antioxidant treatment with NAC could prevent OHB action on DNP-stimulated glucose uptake. Cardiomyocytes were co-incubated with OHB and NAC for 16 h. NAC reacts directly with hydroxyl radicals (•OH) to inactivate them. It is also a scavenger of superoxide anion (O$_2^{-}$) and H$_2$O$_2$ (2). Pretreatment with NAC did not alter DNP action in control cardiomyocytes, and a 2.7-fold increase of DNP-stimulated glucose uptake was observed in these cells ($P < 0.01$) (Fig. 7). Pretreatment with NAC modulated DNP action in OHB-treated cells. Incubation with 75 $\mu$M NAC increased DNP action in OHB-pretreated cardiomyocytes by 58% ($P < 0.05$). However, DNP-mediated glucose uptake was still inhibited by 22% in OHB-treated cells incubated with NAC compared with controls also treated with NAC.

NAC does not restore DNP-stimulated AMPK and p38 MAPK phosphorylation in OHB-treated cardiomyocytes. We next examined the effect of NAC on DNP-mediated activation of the AMPK/p38 MAPK signaling cascade in OHB-pretreated cardiomyocytes. Pretreatment with NAC did not modify AMPK and p38 MAPK concentrations in cardiomyocytes. DNP increased AMPK and p38 MAPK phosphorylation by 1.9- and 4.3-fold in these cells, respectively ($P < 0.05$ and $P < 0.01$, respectively). Thus NAC did not have any effect on DNP-mediated AMPK and p38 MAPK phosphorylation in control cells (Fig. 8, A and B). Consistent with what we observed with H$_2$O$_2$, 75 $\mu$M NAC did not improve DNP action in the OHB-treated group (Fig. 8, A and B). Thus, in NAC-treated cells, DNP-mediated AMPK and p38 MAPK phosphorylation was still totally inhibited by OHB compared with control groups ($P < 0.05$ and $P < 0.01$, respectively).

Discussion

Hyperketonemia is observed during starvation, heart failure, and diabetes. We have demonstrated that chronic exposure to OHB inhibits insulin signaling in cardiomyocytes resulting in diminished glucose uptake in these cells (38, 49). Because glucose represents the major energetic substrate used by the...
heart during metabolic stress, we examined the role of ketone bodies on DNP-mediated glucose uptake in adult cardiomyocytes. Our results show, for the first time, that prolonged exposure to OHB inhibits DNP-stimulated glucose uptake in a time- and concentration-dependent manner in cardiomyocytes. Our data also suggest that two independent mechanisms contribute to OHB action: impaired activation of the AMPK/p38 MAPK signaling cascade and increased ROS generation. This is the first demonstration that ketone bodies inhibit metabolic stress-mediated glucose uptake in the heart.

During diabetic ketoacidosis, ketone body concentrations rise dramatically, reaching plasma levels between 10 and 20 mM (25). In addition, once increased, they remain elevated for more than 12 h after the start of insulin infusion therapy (25). The current study demonstrated that chronic exposure to OHB inhibits the DNP-mediated AMPK/p38 MAPK signaling cascade in cardiomyocytes. To further establish the role of oxidative stress in OHB action, we investigated the effect of H2O2 on the activation of these two enzymes. Our data demonstrated that chronic exposure to H2O2 did not alter DNP-mediated phosphorylation of AMPK and p38 MAPK. Consistent with these results, NAC treatment did not restore AMPK and p38 MAPK phosphorylation in response to DNP in OHB-treated cells, despite improved DNP-stimulated glucose uptake. Thus our data suggest that ROS participate in the regulation of glucose uptake during metabolic stress.

Furthermore, inhibition of p38 MAPK also significantly reduces glucose uptake in response to DNP and ischemia (29, 37, 42, 52). Our results demonstrated that chronic exposure to OHB reduces DNP-mediated AMPK activation in cardiomyocytes. This decrease is paralleled by a complete inhibition of DNP-mediated phosphorylation of ACC and p38 MAPK. Thus inhibition of the AMPK/p38 MAPK signaling pathway may explain, at least in part, the reduction of DNP-stimulated glucose uptake in OHB-treated cardiomyocytes.

Type 1 diabetic patients have impaired insulin- and exercise-stimulated glucose uptake in skeletal muscle (9, 39). Similarly, chronic exposure to ketone bodies impairs both insulin- (49) and DNP-stimulated glucose uptake in cardiomyocytes. Insulin- and metabolic stress-stimulated glucose uptake occurs through activation of two distinct pathways: the insulin receptor substrate-phosphatidylinositol 3-kinase (IRS-PI3K) (21) and AMPK/p38 MAPK (29, 37) signaling cascades. Furthermore, in both cases, only chronic exposure to OHB diminishes glucose uptake. This suggests that the effect of ketone bodies is not due to direct competition between glucose and OHB as energy sources. Furthermore, alteration of two independent signaling cascades by OHB suggests that ketone bodies regulate glucose uptake through a common mechanism. It has been reported that oxidative stress induces insulin resistance (12). Hyperglycemia and dyslipidemia are known factors contributing to increase ROS production (53). However, Jain et al. (20) have reported that hyperketonemic patients present higher lipid peroxidation, a marker of oxidative stress, compared with normoketoneemic diabetic patients. Increased protein oxidation has also been observed in OHB-treated fibroblasts (13) and the skeletal muscle of a congestive heart failure animal model (7). Our data demonstrate that chronic exposure to OHB provokes a 1.9-fold increase in ROS production in cardiomyocytes. Furthermore, our results demonstrated that H2O2 reduces DNP-stimulated glucose uptake in a concentration-dependent manner, suggesting that oxidative stress modulated DNP action in cardiomyocytes. To further investigate the involvement of oxidative stress in the inhibitory effect of OHB, cells were treated with the antioxidant NAC. This treatment has been used successfully to prevent hyperglycemia-induced insulin resistance by decreasing oxidative stress in skeletal muscle (16). Following NAC treatment, we observed a 33% increase in the maximal effect of DNP on glucose uptake in OHB-treated cells. This effect is consistent with the 30% decrease in glucose uptake in H2O2-treated cells. Thus our data suggest that OHB action is mediated, in part, via increased ROS production. To our knowledge, this is the first demonstration that ROS participate in the regulation of glucose uptake during metabolic stress.
cause neither chronic exposure to H₂O₂ nor NAC treatment modulates enzyme activation in response to DNP. This is in contrast to skeletal muscle, where H₂O₂ stimulates AMPK phosphorylation (50) and mediates contraction-stimulated glucose uptake (43). Thus the role of H₂O₂ in the regulation of glucose uptake may be tissue specific. Alternatively, the effect of H₂O₂ on this process may depend on the time of exposure. Similar differences in H₂O₂ action have been observed for glucose uptake. The role of H₂O₂ in the regulation of glucose uptake may be tissue specific. Alternatively, the effect of H₂O₂ on this process may depend on the time of exposure.

Thus, while oxidative stress diminished glucose uptake, this was not paralleled by alteration of the AMPK/p38 MAPK signaling pathway. We, and others, have demonstrated that AMPK inhibition only partially inhibits DNP- and hypoxia-stimulated glucose uptake (29, 37), suggesting that an AMPK-independent pathway needs to be activated to maximally stimulate glucose uptake in the heart. Potentially, OHB-generated oxidative stress may interfere with the activation of this alternative pathway, leading to decreased glucose uptake. While we have shown that OHB does not alter GLUT1 and GLUT4 concentrations in cardiomyocytes (49), inhibition of OHB-mediated glucose uptake could occur through impaired glucose transporter trafficking or fusion/insertion into the plasma membrane. Studies have demonstrated that oxidative stress disrupts insulin-mediated action on actin remodeling, and this may result in impaired GLUT4 translocation (36, 40). A defect at this step would diminish GLUT4 translocation in response to both stimuli. Further studies can examine this possibility.

Our results show that OHB inhibits activation of the AMPK/p38 MAPK signaling pathway in cardiomyocytes, and that this effect is probably not mediated by ROS generation. Diminished AMPK activity has also been reported after a high-fat diet and chronic glucose infusion and in obese Zucker rats (19, 23, 30, 45). These data suggest that AMPK activity is modulated by cellular energy status and reinforce its role as a key modulator of cellular energy balance. Thus AMPK regulation by high-energy supply may be part of a feedback mechanism to limit further uptake of glucose in times of abundant energy sources.

In conclusion, our data demonstrate that prolonged exposure to OHB inhibits DNP-stimulated glucose uptake in a concentration- and time-dependent manner in cardiomyocytes. Ketone bodies alter glucose metabolism by inhibiting activation of the AMPK/p38 MAPK signaling pathway and the generation of oxidative stress. These results suggest that hyperketonemia could have a deleterious effect on cardiac glucose metabolism, especially during metabolic stress.

ACKNOWLEDGMENTS
The editorial assistance of Ovid Da Silva (Research Support Office, Research Centre, CHUM) is acknowledged. We also thank Dr John S. D. Chan for help with ROS measurement.

GRANTS
L. Codenre is a chercheur-boursier supported by the Fonds de la Recherche en Santé du Québec. A. Pelletier is the recipient of a Canadian Diabetes Association/Canadian Institutes of Health Research Doctoral Student Research Award.

REFERENCES
Ketone bodies inhibit DNP action in the heart


