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

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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 AMPK/p38 MAPK activation. 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.

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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 DNase I 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 Bio-Rad (Hercules, CA). All electrophoresis reagents were from Bio-Rad Laboratories (Laval, QC, Canada). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Baie d’Urfe, QC, Canada). The ventricles were minced in KR buffer supplemented with 0.05% collagenase, 15 mM 2,3-butanedione monoxime, 0.2 mg/ml DNase I, 0.1 mM sodium pyrophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 mM sodium fluoride, 10 mM NaCl, and 1 mM sodium orthovanadate. The enhanced chemiluminescence detection system was bought from Amersham Pharmacia Biotech (Baie d’Urfé, QC, Canada). The Bradford protein assay kit was from Bio-Rad (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 insulin, 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 mM 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 NaClO₃ (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 0.5 mM lucigenin (0.5 mM, final concentration). Photon emission was counted every 2.5 min for the first 10 min, after which photoemission 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 underlying 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 normoketonic 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.
production both in control and in OHB-treated cardiomyocytes (Fig. 4B). However, this decrease was significant only in ketone body-treated cells ($P < 0.05$).

$H_2O_2$ 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 $\mu$M) of $H_2O_2$ for 16 h (Fig. 5). Higher $H_2O_2$ concentrations were toxic to the cells (data not shown). Exposure to 5 $\mu$M $H_2O_2$ 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 $\mu$M $H_2O_2$ 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 $H_2O_2$...
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 µM 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 ($P < 0.05$). Consistent with its effect on AMPK phosphorylation, H$_2$O$_2$ did not modulate p38 MAPK phosphorylation in response to DNP, and a 5.9-fold increase of p38 MAPK phosphorylation was observed in response to DNP in H$_2$O$_2$-treated cells ($P < 0.01$) (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 µM 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 µM 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
In response to ischemia, hypoxia, and DNP (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 normoketonic 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.

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 results do not suggest that ROS play a role in the activation of the AMPK/p38 MAPK signaling cascade, be-
cause neither chronic exposure to H$_2$O$_2$ nor NAC treatment modulates enzyme activation in response to DNP. This is in contrast to skeletal muscle, where H$_2$O$_2$ stimulates AMPK phosphorylation (50) and mediates contraction-stimulated glucose uptake (43). Thus the role of H$_2$O$_2$ in the regulation of glucose uptake may be tissue specific. Alternatively, the effect of H$_2$O$_2$ on this process may depend on the time of exposure. Similar differences in H$_2$O$_2$ action have been observed for contrast to skeletal muscle, where H$_2$O$_2$ stimulates AMPK of H$_2$O$_2$ on this process may depend on the time of exposure. Glucose uptake may be tissue specific. Alternatively, the effect of AMPK/p38 MAPK signaling pathway and abnormal localization of insulin signaling molecules, such as PI3K (36, 40, 44).

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, 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.

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