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β-Hydroxybutyrate inhibits insulin-mediated glucose transport in mouse oxidative muscle

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Yamada T, Zhang SJ, Westerblad H, Katz A. β-Hydroxybutyrate inhibits insulin-mediated glucose transport in mouse oxidative muscle. *Am J Physiol Endocrinol Metab* 299: E364–E373, 2010. First published June 1, 2010; doi:10.1152/ajpendo.00142.2010.—Blood ketone body levels increase during starvation and untreated diabetes. Here we tested the hypothesis that ketone bodies directly inhibit insulin action in skeletal muscle. We investigated the effect of D,L-β-hydroxybutyrate (BOH; the major ketone body in vivo) on insulin-mediated glucose uptake (2-deoxyglucose) in isolated mouse soleus (oxidative) and extensor digitorum longus (EDL; glycolytic) muscle. BOH inhibited insulin-mediated glucose uptake in soleus (but not in EDL) muscle in a time- and concentration-dependent manner. Following 19.5 h of exposure to 5 mM BOH, insulin-mediated (20 mU/ml) glucose uptake was inhibited by ~90% (substantial inhibition was also observed in 3-*O*-methylglucose transport). The inhibitory effect of BOH was reproduced with D- but not L-BOH. BOH did not significantly affect hypoxia- or AICAR-mediated (activates AMP-dependent protein kinase) glucose uptake. The BOH effect did not require the presence/utilization of glucose since it was also seen when glucose in the medium was substituted with pyruvate. To determine whether the BOH effect was mediated by oxidative stress, an exogenous antioxidant (1 mM tempol) was used; however, tempol did not reverse the BOH effect on insulin action. BOH did not alter the levels of total tissue GLUT4 protein or insulin-mediated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 but blocked insulin-mediated phosphorylation of protein kinase B by ~50%. These data demonstrate that BOH inhibits insulin-mediated glucose transport in oxidative muscle by inhibiting insulin signaling. Thus ketone bodies may be potent diabetogenic agents in vivo.

ketone bodies; soleus; extensor digitorum longus; glucose transporter 4

UNCONTROLLED DIABETES IS ASSOCIATED with increased oxidation of free fatty acids (FFA), which results in accelerated ketone body [acetoaceate (AcAc), β-hydroxybutyrate (BOH), and acetone] production by the liver (17). On the one hand, ketone bodies can lead to acidosis (ketoacidosis) if excessive levels are reached in blood (18). On the other hand, they can be beneficial by serving as alternative energy substrates for key extrahepatic oxidative tissues (e.g., heart and brain) (17, 24, 34). Short-term starvation is another condition associated with elevated rates of FFA oxidation and marked elevations in blood ketone body levels (8). A common denominator between uncontrolled diabetes and starvation is insulin resistance (6, 9, 10), defined as diminished glucose transport by skeletal muscle in response to insulin. The elevated levels of FFA during uncontrolled diabetes and starvation can contribute to the insulin resistance (27). Indeed, it is well documented that FFA inhibit insulin-mediated glucose transport in skeletal muscle (1, 27, 32, 39). However, whether ketone bodies can cause insulin resistance in skeletal muscle is not clear.

Generally, early studies showed that acute exposure of rodent skeletal muscle preparations to AcAc or BOH did not alter insulin-mediated glucose uptake in a noteworthy fashion (4, 27). Similarly, short-term AcAc or BOH infusion did not alter whole body glucose disposal during hyperinsulinemia in pigs or humans (5, 21), where skeletal muscle accounts for ~90% of insulin-mediated glucose disposal (10). Interestingly, subsequent studies demonstrated that, whereas acute exposure (4 h) to BOH did not alter insulin-mediated glucose uptake in cardiomyocytes, prolonged exposure (16 h) resulted in marked inhibition (31). These findings suggest that earlier studies with skeletal muscle reported a lack of effect of ketone bodies, owing to an insufficient exposure period. Indeed, starvation and untreated diabetes are associated with prolonged exposure to ketone bodies.

The purpose of the present investigation was to study the effect of BOH on glucose transport in isolated mouse muscle preparations. We demonstrate that prolonged exposure of mouse oxidative muscle to physiological concentrations of BOH diminishes insulin-mediated glucose uptake. Furthermore, the effect of BOH is mediated by interference with insulin signaling and not by altering the amount of GLUT4 protein levels.

RESEARCH DESIGN AND METHODS

Materials and animals. 2-Deoxy-D-[1,2-^{3}H]glucose (2-DG), 3-*O*-[^{3}H]methylglucose (3-OMG), and [carboxy-^{14}C]inulin were from Amersham Biosciences. D,L-BOH, sodium salt, sodium (S)-3-hydroxybutyrate (L-BOH), (R)-(−)-3-hydroxybutyric acid (D-BOH), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol), and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) were from Sigma. Human insulin (Actrapid) was from Novo Nordisk. Antibodies against GLUT4 and GLUT1 were from Chemicon, and antibody against dihydropyridine receptor (DHPR) was from Abcam. Antibodies against the β-subunit of the insulin receptor (IR) were from Millipore, against tyrosine-phosphorylated IR (p-IR)(pY972) from Invitrogen, against insulin receptor substrate-1 (IRS-1) from Chemicon, and against tyrosine-phosphorylated IRS-1(p-IRS-1; pY612) from Invitrogen. Antibodies against malondialdehyde (MDA), nitrotyrosine, and phosphorylated protein kinase Cδ (p-PKCδ) were from Academy Bio-Medical, Upstate Biotechnology, and Cell Signaling Technology, respectively.

Male NMRI mice weighing 25–30 g were housed at room temperature with a 12:12-h light-dark cycle. Food and water were provided ad libitum. The mice were purchased from B & K Universal (Sollentuna, Sweden). Animals were euthanized by rapid cervical dislocation, and the soleus and extensor

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digitorum longus (EDL) muscles were isolated and incubated in Tyrode solution with the following composition (in mM): 121 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 24 $NaHCO_3$, 5 glucose, and 1% bovine serum albumin (fatty acid free), unless indicated otherwise. The air phase in the vial containing the Tyrode solution was continuously gassed with 95% O_2-5% CO_2, resulting in a pH of 7.4 in the solution. EDL and soleus muscle weights ranged from 11 to 19 mg wet wt. All procedures were approved by the Stockholm North Ethics Committee.

Glucose uptake/transport. Muscles were transferred to vials containing 1.5 ml of Tyrode solution with or without additions, incubated in a shaking water bath (110 oscillations/min, 25°C) for a total of 19.5 h (unless stated otherwise), and frozen in liquid nitrogen. The pH of the medium averaged 7.48 (7.42, 7.54; $n = 2$) at the beginning of the incubation and 8.12 (8.09, 8.15) at the end of the 19.5-h incubation period; BOH did not affect the pH at either the beginning (mean = 7.45, 7.44, and 7.46) or the end (mean = 8.12, 8.12, and 8.13). No medium changes occurred during the incubations. [It should be noted that glucose transport in isolated rodent muscles is constant at medium pH, ranging from 6.8 to 8.3 (28).] To control for the osmotic effect of BOH, 5 mM NaCl was always added to the Tyrode solution bathing the contralateral muscle. The low temperature was used to enhance viability of the muscle. We have shown previously that muscle viability is well maintained under such conditions, as judged by the ability to generate tetanic force (29). Furthermore, the extracellular space in muscles incubated for 19.5 h was virtually identical to those incubated for only 2 h (all muscles had values of 0.2–0.3 ml/g wet wt; BOH did not affect extracellular space under any condition studied). In all glucose uptake experiments, radiolabeled 2-DG (1 mCi/mmol 2-DG, 1 mM 2-DG; or 0.2 mCi/mmol glucose, 5 mM glucose) and inulin (1 μCi/ml medium) were present during the last 30 min of incubation, as described elsewhere (30). For measurement of glucose transport, radiolabeled 3-OMG (1 mCi/mmol 3-OMG, 1 mM 3-OMG) was present during the last 15 min of incubation (30). To assess the effects of BOH on insulin-mediated glucose uptake/transport, insulin (20 mU/ml) was added 90 min before freezing. Other modes of glucose uptake activation were also studied to assess specificity of BOH action. To examine the effects of BOH on hypoxia-induced glucose uptake, muscles were continuously gassed with 95% N_2-5% CO_2 during the last 90 min of incubation. AICAR is converted in the cell to AICAR monophosphate, which is an AMP mimetic that activates AMP-activated protein kinase (AMPK) and AMPK kinase (16). To examine the effects of BOH on AICAR-induced glucose uptake, 2 mM AICAR was added 110 min before freezing. To examine the role of reactive oxygen species in BOH effects on glucose uptake, muscle was incubated with 1 mM tempol, a stable, membrane-permeable nitroxide that acts as a scavenger of superoxide (15).

For analysis of 2-DG uptake or 3-OMG transport, frozen muscles were added to preweighed Eppendorf tubes containing 0.5 ml of 1 N NaOH. The muscle was weighed and then digested at 70°C for 15 min. The tubes were cooled on ice and centrifuged at 23,000 *g* for 5 min. Aliquots of the supernatant were added to scintillation cocktail and counted for ^{14}C and ^{3}H, as described earlier (30).

D-BOH dehydrogenase. For analysis of D-BOH dehydrogenase activity (which converts D-BOH to AcAc), muscles were isolated and frozen in liquid N_2. Muscles were homogenized with a motor-driven ground glass homogenizer in ice-cold buffer (20 μl/mg wet weight) consisting of (in mM) 50 TEA, 1 EDTA, 2 $MgCl_2$, 2 dithiothreitol, and 0.05% Triton X (vol/vol), pH 7.5. The homogenate was centrifuged for 30 s at 1,400 *g*. The supernatant (50 μl) was assayed by following the D,L-3-BOH-dependent (20 mM) formation of NADH on a spectrophotometer at 340 nm at room temperature (3). Two types of blanks were used: *1*) homogenization buffer and *2*) reaction mixture lacking substrate. Both blanks changed insignificantly during incubation (60 min). Reaction rates were linear with respect to extract volume and assay duration (data not shown).

Western blots. For Western blot analyses, muscles were incubated in Tyrode solution with or without BOH for 19.5 h and then frozen in liquid nitrogen. Insulin was present during the last 90 min in half of the samples. Blots were performed for GLUT4, GLUT1, IR, p-IR, IRS-1, p-IRS-1, DHPR, MDA, nitrotyrosine, and p-PKCδ. Briefly, frozen muscles were homogenized in ice-cold lysis buffer (40 μl/mg wet wt) consisting of (in mM) 20 HEPES (pH 7.6), 150 NaCl, 5 EDTA, 25 KF, 1 Na_3VO_4, 20% glycerol (vol/vol), 0.5% Triton X-100 (vol/vol), and 1 tablet protease inhibitor cocktail (Roche) per 50 ml. Lysates were cleaned by centrifugation for 10 min at 1,000 *g*. The protein content was determined using the Bradford assay (Bio-Rad). Aliquots of the lysate (20 μg) were then subjected to SDS-PAGE (12% Bis-Tris gels; Invitrogen). Proteins were transferred onto polyvinylidine fluoride membranes. Membranes were blocked in 5% (wt/vol) nonfat milk and Tris-buffered saline containing 0.05% Tween-20 followed by incubation with primary antibody and made up in 5% (wt/vol) nonfat milk (GLUT4, 1:5,000; IR, 1:200; p-IR, 1:1,000; IRS-1, 1:200; p-IRS-1, 1:1,000; MDA and nitrotyrosine, 1:1,000; p-PKCδ and DHPR, 1:500) overnight at 4°C. Membranes were then washed and incubated for 1 h at room temperature with secondary antibody (donkey anti-rabbit or donkey anti-mouse, 1:5,000). Immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal; Pierce). Band densities were analyzed with Image J (http//rsb.info.nih.gov/).

Phosphorylation of protein kinase B. Aliquots of supernatant protein (prepared as for Western blots; see above) were used for analysis of phosphorylated protein kinase B (p-PKB) and total PKB using the PathScan ELISA Kit (7160 and 7170, respectively; Cell Signaling Technology), following the supplier's test procedures.

Statistics. Significant differences between means were determined with Student's *t*-test for paired samples. $P < 0.05$ was regarded as statistically significant. Values are presented as means ± SE.

RESULTS

Effect of BOH on basal and insulin-stimulated glucose uptake. Exposure of soleus muscle to 5 mM BOH for 2 h did not affect glucose uptake in the absence (basal) or presence of insulin (Fig. 1*A*). However, following 9.5 h of exposure to BOH, the response to insulin was decreased by ~50% and almost completely abolished after 19.5 h. The insulin effect on 2-DG uptake in the absence of BOH appeared to decrease as a

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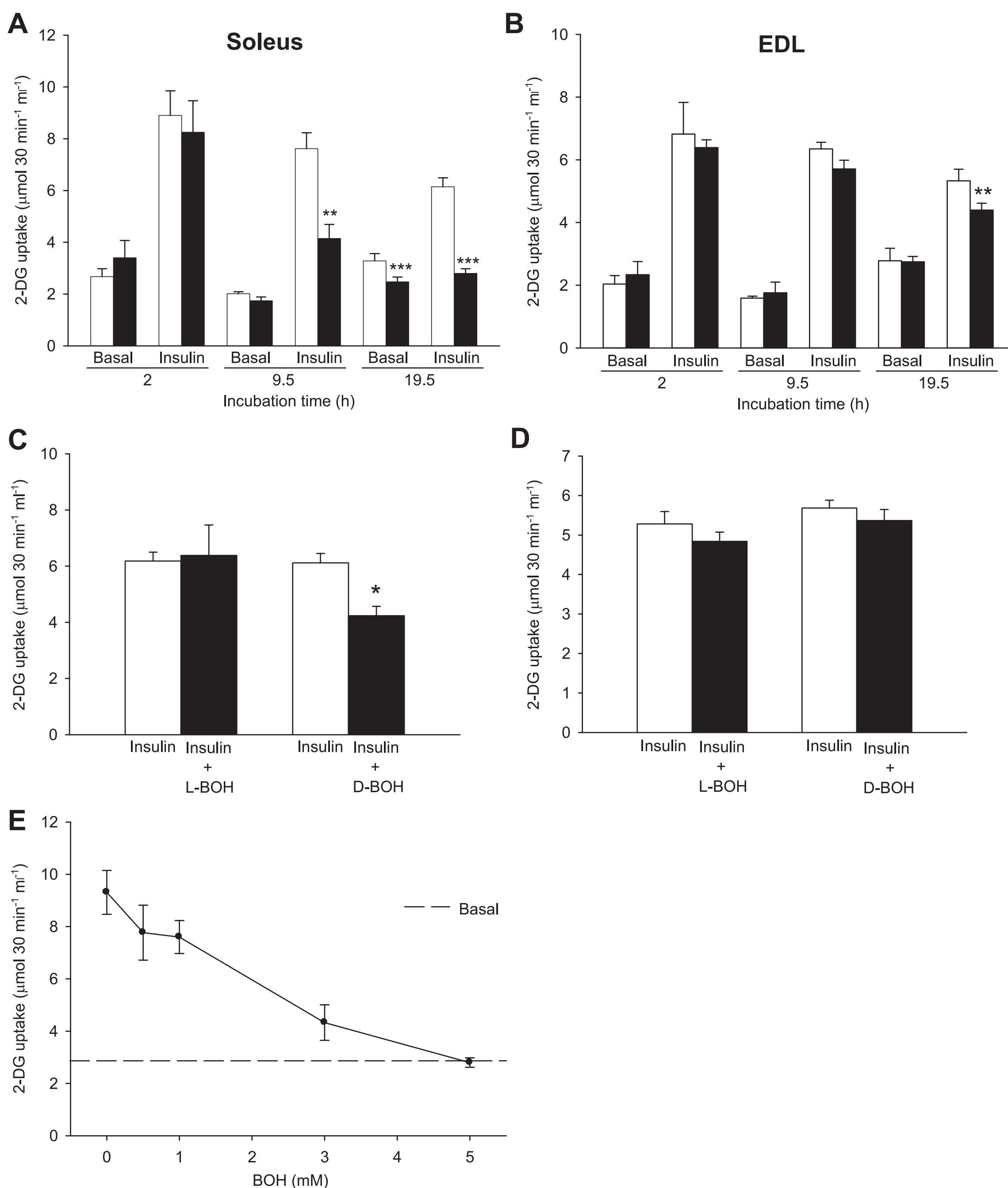

Fig. 1. Effect of β-hydroxybutyrate (BOH) on glucose transport in soleus and extensor digitorum longus (EDL) muscles. Basal and insulin-mediated 2-deoxy-D-[1,2-^{3}H]glucose (2-DG) uptake of soleus (*A*) and EDL (*B*) muscles with or without 5 mM BOH for 2, 9.5, or 19.5 h. Open bars, control; filled bars, D,L-BOH. Insulin-mediated 2-DG uptake of soleus (*C*) and EDL (*D*) muscles with or without 5 mM sodium-3-hydroxybutyrate (L-BOH) or (R)-(−)-3-hydroxybutyric acid (D-BOH) for 19.5 h. Open bars, control (insulin); filled bars, insulin + L-BOH or D-BOH as indicated. Data are presented as means ± SE for 5–6 muscles in each group. *E*: insulin-mediated 2-DG uptake of soleus muscles with 0, 0.5, 1, 3, or 5 mM BOH for 19.5 h. Dashed line indicates 2-DG uptake under basal conditions (no insulin). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control.

function of time. Whether this is a real phenomenon or attributable to experimental variability is unclear. Noteworthy is that following 19.5 h of incubation in pyruvate in the absence of BOH, the insulin effect on 2-DG uptake was, in fact, relatively greater (see Fig. 5*A*) than after 2 h of incubation (Fig. 1*A*).

The effect on insulin action was concentration dependent, with half-maximal inhibition occurring at a BOH concentration of ~2 mM (Fig. 1*E*). On the other hand, in EDL muscle, BOH did not affect glucose uptake after 2 or 9.5 h and only slightly decreased uptake in the presence of insulin after 19.5 h (Fig. 1*B*). Thus BOH has negative effects on glucose uptake primarily in oxidative muscle. In all subsequent experiments, muscles were incubated for 19.5 h in the absence or presence of 5 mM BOH.

BOH exists as D- and L-stereoisomers. To determine whether differences between the two ligands exist, muscles were incu-

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bated in 5 mM D- or L-BOH. Only D-BOH showed a negative effect on insulin-mediated glucose uptake, and this was observed only in soleus (Fig. 1*C*) and not in EDL muscle (Fig. 1*D*). Thus, D-BOH, which is the primary stereoisomer in vivo (33), is the inhibitory moiety.

D-BOH is the substrate that is recognized by D-BOH dehydrogenase. To determine whether the differential effects of BOH on EDL and soleus muscles could derive from differences in the capacity to utilize BOH, we measured the activity of D-BOH dehydrogenase. Activity in the EDL was detectable but low (0.03 ± 0.001 $\mu mol \cdot min^{-1} \cdot g$ wet wt^{-1}; $n = 3$), whereas a robust activity was measured in the soleus (0.44 ± 0.07; $n = 3$). Heart, which is very oxidative and is known to have a high capacity to utilize BOH, had an activity of 2.5 $\mu mol \cdot min^{-1} \cdot g$ wet wt^{-1} ($n = 1$).

Effect of BOH on hypoxia- and AICAR-mediated glucose transport. To study the specificity of the BOH effect, other modes of glucose uptake were also studied. Hypoxia and AICAR increase glucose uptake in skeletal muscle via insulin-independent pathways (12). Neither hypoxia- nor AICAR-mediated glucose uptake was significantly affected by BOH (Fig. 2, *A* and *B*).

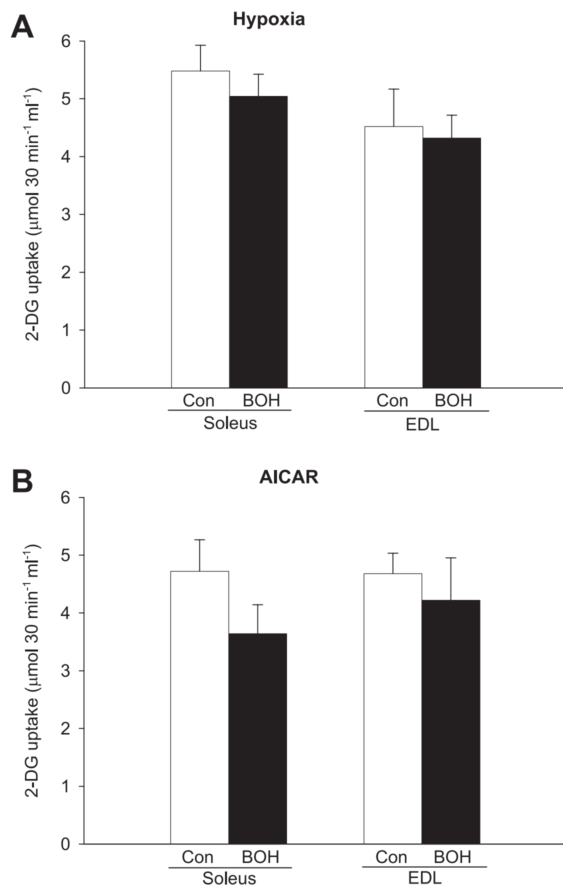

Fig. 2. Effect of BOH on hypoxia- or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-mediated glucose transport in soleus and EDL muscles. Hypoxia- (*A*) or AICAR-mediated 2-DG uptake (*B*) of soleus and EDL muscles. Open bars, control (Con); filled bars, 5 mM D,L-BOH. Muscles were incubated for 19.5 h and exposed to hypoxia or AICAR during the last 90 and 110 min, respectively. 2-DG uptake measurements occurred during the last 30 min of incuation. Data are presented as means ± SE for 5 muscles in each group.

Effect of BOH on total GLUT4 expression, insulin signaling, and glucose transport. Since the effects of BOH on insulin-mediated glucose uptake were most marked at 19.5 h, the latter incubation duration was used in subsequent experiments to investigate how BOH exerts its effects. The reduction in insulin-stimulated glucose uptake by BOH could result from decreases in the expression of GLUT4 transporters or decreases in activity of the signaling molecules that recruit GLUT4 transporters to surface membranes; the latter case would indicate that BOH was specifically inhibiting the transport of glucose as opposed to its metabolism, i.e., phosphorylation. To address these possibilities, we first determined the total GLUT4 protein levels in control and BOH-treated muscles. Figure 3, *A* and *B*, shows representative Western blots of GLUT4 content in soleus and EDL muscles. The densitometric evaluation of the blot revealed that the total GLUT4 expression was not affected by BOH under any condition studied (Fig. 3). GLUT1 expression was also not affected by BOH (control soleus plus insulin = 4,129 ± 584 arbitrary units, BOH soleus plus insulin = 4,142 ± 405; $n = 4$ for each group).

Insulin induced marked increases in the levels of p-IR and p-IRS-1 in soleus muscle, and BOH did not alter the response (Fig. 4, *A* and *B*). In contrast, BOH inhibited insulin-mediated phosphorylation of PKB in soleus muscle by ~50% (Fig. 4C). Again, BOH did not alter the insulin effect in EDL muscle (Fig. 4*D*). Insofar as insulin signaling was diminished by BOH in soleus muscle, one would expect a similar effect of BOH on glucose transport and GLUT4 translocation to surface membranes. Indeed, BOH also inhibited insulin-mediated glucose (3-OMG) transport in soleus but not in EDL muscle (Fig. 4, *E* and *F*). Moreover, 3-OMG transport in response to insulin also reflects the degree of insulin-mediated GLUT4 translocation to surface membranes in isolated rodent muscle preparations (19, 36). Thus the results indicate that BOH inhibits insulin-mediated glucose transport in soleus muscle by interfering with insulin signaling and not by altering the total availability of GLUT4 transporters.

Role of glucose metabolism, reactive oxygen species/reactive nitrogen species production, and p-PKCδ in BOH-induced insulin resistance. To assess whether BOH-mediated inhibition of insulin action requires the presence or metabolism of glucose, muscles were incubated with 2 mM pyruvate instead of 5 mM glucose in the Tyrode solution. In soleus muscles, the inhibition of insulin-mediated sugar uptake also occurred when glucose was excluded from the medium (Fig. 5*A*). Again, no noteworthy effect was seen in EDL muscle (Fig. 5*B*). (Note that sugar uptake was measured with 1 mM 2-DG; hence, the lower rates of glucose uptake compared with 5 mM glucose elsewhere.)

BOH and AcAc induce reactive oxygen species (ROS) production in isolated cardiomyocytes and erythrocytes (14, 25), and excessive ROS production has been implicated in insulin resistance (7, 13). Therefore, we investigated whether ROS production plays a role in ketone body action by using the antioxidant tempol. Soleus muscles were coincubated with BOH and tempol for 19.5 h. If BOH decreases the effect of insulin on glucose uptake (see Fig. 1) via increased ROS production, then tempol would be expected to increase glucose uptake in the presence of insulin and BOH. However, incubation with tempol did not reverse the BOH-mediated inhibition

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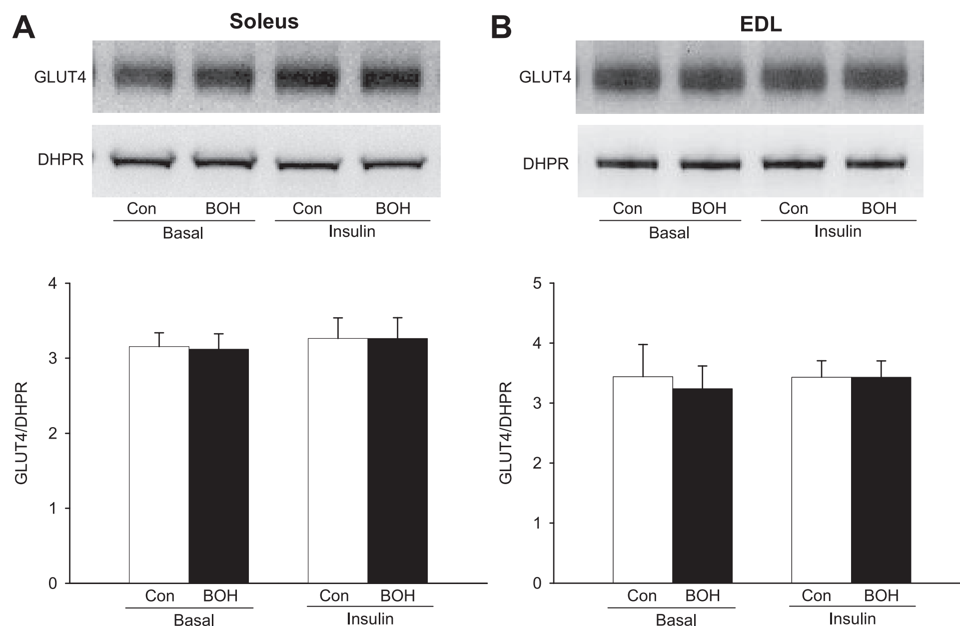

Fig. 3. Effect of BOH on total GLUT4 contents in soleus and EDL muscles. Representative immunoblots of GLUT4 in soleus (*A*) and EDL (*B*) muscles (*top*) and densitometric analyses (*bottom*). Anti-dihydropyridine receptor (DHPR) antibody was used as a loading control. Open bars, Con; filled bars, 5 mM D,L-BOH (BOH). Data are presented as means ± SE for 5–6 muscles in each group.

of insulin-mediated glucose uptake in soleus muscle (Fig. 5*C*), nor did it affect transport in EDL muscle (Fig. 5*D*).

To more directly assess whether BOH induced ROS/reactive nitrogen species (RNS)-mediated alterations in protein structure, we used antibodies against MDA-protein adducts and nitrotyrosine. BOH did not affect MDA-protein adducts (Fig. 6*A*) or nitrotyrosine content in soleus (Fig. 6*C*) or EDL muscle (Fig. 6, *B* and *D*). These data do not support a role for excessive production of ROS/RNS in the inhibitory effect of BOH on insulin action.

Recently, activation (phosphorylation) of PKCδ was implicated in inhibition of insulin-mediated phosphorylation of PKB (35). Therefore, experiments were performed to investigate whether BOH altered the phosphorylation state of PKCδ. BOH did not alter p-PKCδ levels either in the absence of insulin (control = 0.88 ± 0.09, BOH = 0.87 ± 0.07; $n = 5$; units are density of p-PKCδ/DHPR) or in its presence (control = 1.05 ± 0.08, BOH = 0.97 ± 0.04; $n = 3$).

DISCUSSION

The major findings of the present study are that *1*) prolonged exposure to physiological concentrations of BOH diminishes insulin- but not AICAR- or hypoxia-mediated glucose uptake in oxidative muscle, *2*) the inhibitory effect of BOH is associated with diminished PKB phosphorylation, and *3*) the development of insulin resistance by BOH is not associated with excessive ROS/RNS production.

Plasma ketone body concentrations vary greatly depending on the nutritional or pathological state. In healthy individuals, the BOH concentration is usually <0.2 mM, but during prolonged starvation and uncontrolled diabetes it can increase to 7–10 mM (11, 23). Thus the concentration of BOH used in the present study (5 mM) is clearly within the range observed in vivo.

The finding that the BOH effect on insulin-mediated glucose uptake was time dependent and expressed primarily in oxidative muscle may explain the general inability of earlier studies to document inhibitory effects of ketone bodies in skeletal muscle (see INTRODUCTION), although one study did show that AcAc inhibited the insulin-mediated removal of glucose from the medium by isolating rat soleus muscle (20). The former studies either employed short-time exposure to ketones or measured whole body glucose disposal in response to insulin, which will reflect both oxidative and glycolytic muscles (4, 5, 21, 27). However, our findings are consistent with those observed in isolated rat cardiomyocytes (highly oxidative cells), where the decreased insulin effect on glucose uptake was also time dependent (31). Indeed, there are, despite the differences in preparations (cardiomyocytes vs. intact skeletal muscle preparations), several other similarities in the results. For example, BOH did not alter GLUT4 protein levels or insulin-mediated phosphorylation of the insulin receptor and did inhibit insulin-mediated PKB phosphorylation (26, 31). On the other hand, there were some differences as well. Thus, in cardiomyocytes, BOH inhibited insulin-mediated phosphorylation of IRS-1 and appeared to induce oxidative stress (25, 26), which differed from the current findings.

Although the time course of reversal of the BOH effect was not addressed in the current study, this was examined earlier in cardiomyocytes. There it was shown that ~8 h were required to normalize insulin action following removal of BOH (31).

How prolonged exposure to BOH results in insulin resistance is not clear. In skeletal muscle, neither ROS/RNS nor phosphorylation of PKCδ appears to be involved. But it seems likely that the mitochondrial enzyme D-BOH dehydrogenase (which converts D-BOH to AcAc) is required to mediate the BOH effect. This is supported by the observation that L-BOH (which is not recognized by D-BOH dehydrogenase as a substrate) was ineffective in inducing insulin resistance, whereas D-BOH was effective. Moreover, BOH had no noteworthy effect on insulin action in EDL (glycolytic) muscle, which can be attributed to either the low amount or lack of detectable D-BOH dehydrogenase activity in glycolytic mammalian muscle (3, 37, 38). This was also the case in the present study, where activity was low in the

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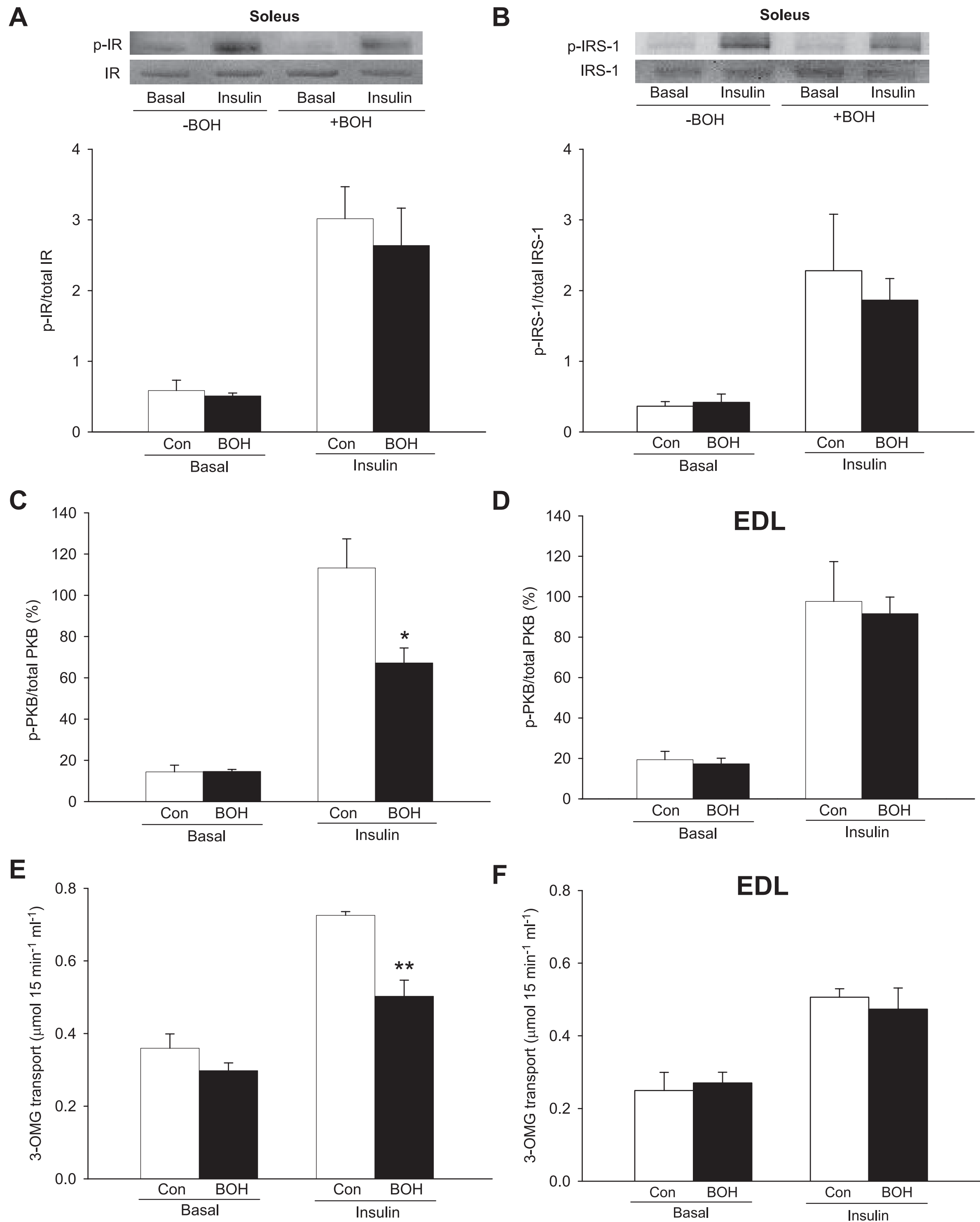

Fig. 4. Effect of BOH on insulin signaling and 3-*O*-[^{3}H]methylglucose (3-OMG) transport in soleus and EDL muscles. Representative immunoblots of phosphorylated insulin receptor (p-IR; *A*) and insulin receptor substrate-1 (p-IRS-1; *B*) in soleus muscles (*top*) and densitometric analyses (*bottom*). Representative immunoblots of total IR (*A*) and total IRS-1 (*B*) in soleus muscles are also shown. ELISA analysis (spectrophotometric) of the proportion of phosphorylated to total PKB in soleus (*C*) and EDL (*D*) muscles. Total PKB was not affected by BOH under any condition studied (soleus: basal control = 0.26 ± 0.02, basal BOH = 0.26 ± 0.01, insulin control = 0.31 ± 0.01, insulin BOH = 0.31 ± 0.01; EDL: basal control = 0.25 ± 0.02, basal BOH = 0.24 ± 0.01, insulin control = 0.29 ± 0.03, insulin BOH = 0.32 ± 0.01; values are net changes in absorbance). 3-OMG transport in soleus (*E*) and EDL (*F*) muscles. Data are presented as means ± SE for 5–6 muscles in each group. Open bars, Con; filled bars, 5 mM D,L-BOH. *$P < 0.05$; **$P < 0.01$ vs. control.

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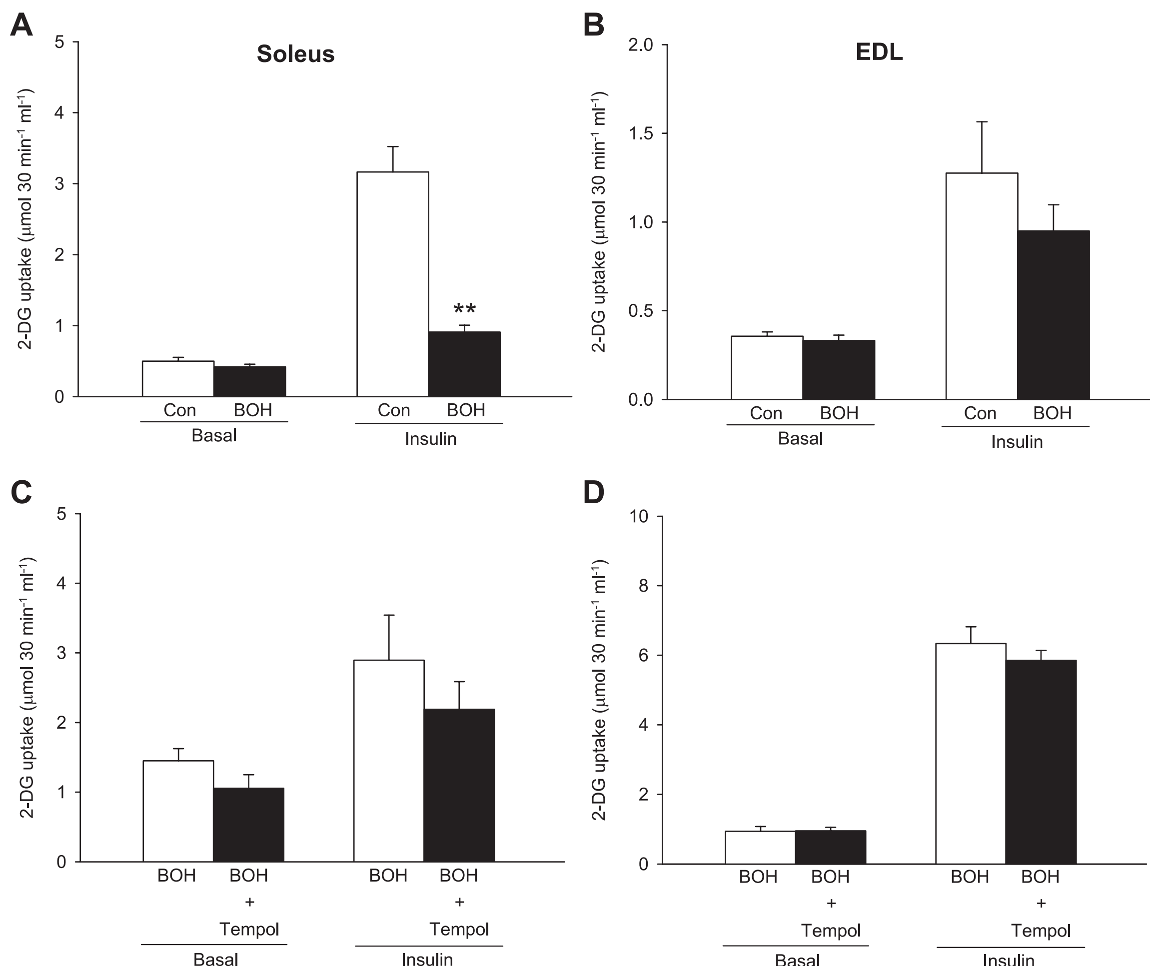

Fig. 5. Effect of glucose metabolism and antioxidants on BOH action. Basal and insulin-mediated 2-DG uptake of soleus (*A*) and EDL (*B*) muscles with or without 5 mM BOH for 19.5 h. Muscles were incubated with 2 mM pyruvate instead of 5 mM glucose in the Tyrode solution. In these experiments, the concentration of 2-DG in the medium was 1 mM; hence, the lower rates of 2-DG uptake. Data are presented as means ± SE for 5 muscles in each group. Open bars, Con; filled bars, 5 mM D,L-BOH (BOH). $^{**}P < 0.01$ vs. control. Basal and insulin-mediated 2-DG uptake of soleus (*C*) and EDL (*D*) muscles exposed to 5 mM BOH for 19.5 h with or without 1 mM tempol. Here (*C* and *D*) the concentration of glucose in the medium was 5 mM. Note that insulin-mediated 2-DG uptake in the absence of BOH and tempol (*C*) is ~6 μmol·30 min^{-1}·ml^{-1}. Data are presented as means ± SE for 5–6 muscles in each group. Open bars, 5 mM D,L-BOH; filled bars, 5 mM D,L-BOH with 1 mM tempol (BOH + tempol).

EDL and about 15-fold higher in soleus. Thus mitochondrial metabolism of BOH is involved in transducing the inhibitory effect of BOH on insulin-mediated glucose transport in oxidative muscle.

2-DG glucose uptake technically reflects the sum of glucose transport and phosphorylation by hexokinase. In an earlier study, it was shown that exposure of isolated rat soleus muscle to AcAc decreased insulin-mediated glucose uptake, with no effect in EDL muscle (i.e., similar to the findings of the present study) (20). These investigators provided evidence supporting the idea that AcAc resulted in the inhibition of glucose phosphorylation (i.e., hexokinase) and that this contributed to the negative effect of BOH on insulin-mediated removal of glucose from the incubation medium. These authors demonstrated that AcAc significantly increased muscle citrate levels and that this was associated with large increases in glucose 6-phosphate levels, supporting the involvement of the Randle hypothesis (citrate inhibits phosphofructokinase, resulting in an accumulation of glucose 6-phosphate, which will inhibit hexokinase and, hence, glucose uptake). The latter hypothesis is often used to explain how FFA result in inhibition of glucose phosphorylation and glucose transport (23, 27). Noteworthy, however, is that Maizels et al. (20) also demonstrated that incubating soleus muscle in glucose-free medium containing AcAc and insulin did not result in an accumulation of citrate or glucose 6-phosphate. The current experiments demonstrate that, even when soleus muscle is incubated in glucose-free medium, insulin-mediated glucose (2-DG) uptake is still abolished by BOH (see Fig. 5), which indicates that BOH does not affect glucose uptake via the Randle hypothesis. Moreover, the experiments with 3-OMG demonstrate that BOH specifically interferes with the transport step, most likely by interfering with insulin signaling (as judged by diminished insulin-mediated PKB phosphorylation).

In this context, it is of interest to compare the effects of FFA and BOH on insulin-mediated glucose transport. Although FFA can exert inhibitory effects on insulin-mediated glucose transport within 1 h (27), there is usually a time-dependent component that can continue for up to 18 h in isolated rat soleus muscle preparations (1, 32). Thus, in this

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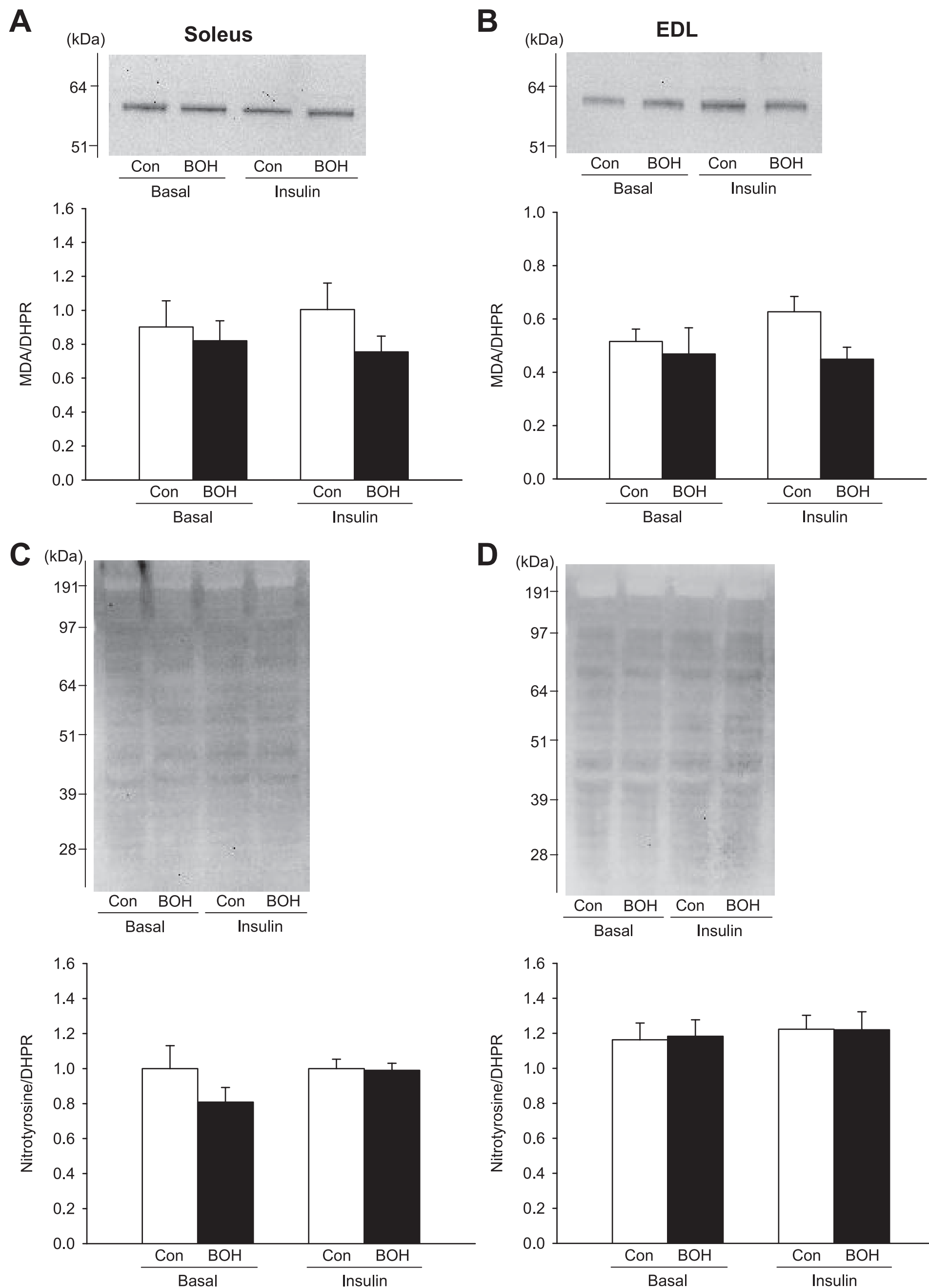

Fig. 6. Effect of BOH on malondialdehyde (MDA)-protein adducts and nitrotyrosine formation in soleus and EDL muscle. Representative immunoblots of MDA-protein adducts in soleus (*A*) and EDL (*B*) muscles (*top*) and densitometric analyses (*bottom*). There were also a number of additional, albeit very weak, bands seen in the MDA blots. However, these were not affected by BOH under any condition (data not shown). Exposure of isolated mouse glycolytic muscle to 100 μM *tert*-butylperoxide results in a marked increase of band intensity [positive control (2)]. Representative immunoblots of nitrotyrosine in soleus (*C*) and EDL (*D*) muscles (*top*) and densitometric analyses (*bottom*). In experiments from separate gels, exposure of extract from mouse oxidative muscle to ≥5 mM 3-morpholinosydnonimine hydrochloride (a peroxynitrite donor) resulted in a marked increase in band intensity (positive control; data not shown). Data are presented as means ± SE for 5–6 muscles in each group. Values are normalized to DHPR content. Open bars, Con; filled bars, 5 mM D,L-BOH.

respect, the effects of FFA and BOH are similar. Earlier, it was shown that FFA and ketone bodies inhibit glycolysis, and this was believed to be a consequence of elevated citrate levels (23, 27). However, it appears unlikely that the citrate mechanism applies to the BOH effect in the present study (see above).

The data in the present study suggest that BOH exerts its inhibitory effect on insulin action by altering mitochondrial metabolism that ultimately results in the inhibition of insulin signaling in oxidative muscle. The fact that the time dependency of the BOH effect is so prolonged leads us to speculate that the synthesis/degradation of a specific protein(s) is involved. However, it is unlikely that BOH has a generalized negative effect on protein synthesis/degradation, as evidenced by the finding that the expression of GLUT4 and insulin-signaling proteins was unaffected. Similarly, the expression of contractile proteins and ion channels (e.g., DHPR; see Fig. 3) appeared to be unaffected, as judged by lack of a BOH effect on muscle function. Thus 18-h exposure to 5 mM BOH did not negatively affect tetanic force in isolated muscles measured at 70 Hz (control = 152 ± 10 mN, BOH = 162 ± 34 mN; $n = 3$).

The findings of this study raise the question of why BOH can be simultaneously beneficial (as a substrate) and detrimental (causing insulin resistance) in the same tissue (skeletal muscle). The answer may be considered within the context of the thrifty gene hypothesis (22). The essential idea behind this hypothesis is that during the period of hunters and gatherers, humans who were exceptionally efficient in the intake or

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utilization of foods had a better chance of surviving periods of famine. During starvation or calorie restriction, muscle insulin resistance would be beneficial by channeling circulating glucose to critical tissues (e.g., brain). Ketones, together with FFA, would then supply the muscle with necessary substrate while at the same time inhibit glucose uptake. However, during the hunter/gatherer period there were larger fluctuations in nutrient availability, and people were more physically fit. Thus the potential negative effects of ketones were probably minimal. Nowadays nutrient availability is essentially unlimited, and people are generally less physically fit, especially in Western societies. Therefore, the danger of ketosis would be relegated mostly to uncontrolled diabetes, calorie restriction, or various ketogenic diet therapies. These events can last for weeks, months, or longer and therefore contribute to the symptoms seen in insulin-resistant states (e.g., the metabolic syndrome and related conditions). This would make ketone bodies bona fide physiological diabetogenic agents. Thus deciphering the mechanism behind ketone body-induced insulin resistance could prove useful in furthering our understanding of insulin resistance and associated diseases (e.g., type 2 diabetes).

In conclusion, our data demonstrated that prolonged exposure to BOH inhibits insulin-mediated glucose uptake in a concentration- and time-dependent manner in oxidative muscle. The BOH effect appears to require mitochondrial metabolism that ultimately results in the inhibition of insulin signaling. Thus ketone bodies may be potent diabetogenic agents in vivo.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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