Inhibition of xanthine oxidase reduces hyperglycemia-induced oxidative stress and improves mitochondrial alterations in skeletal muscle of diabetic mice

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Bravard A, Bonnard C, Durand A, Chauvin M, Favier R, Vidal H, Rieusset J. Inhibition of xanthine oxidase reduces hyperglycemia-induced oxidative stress and improves mitochondrial alterations in skeletal muscle of diabetic mice. Am J Physiol Endocrinol Metab 300: E581–E591, 2011. First published January 11, 2011; doi:10.1152/ajpendo.00455.2010.—Reactive oxygen species (ROS) have been widely implicated in the pathogenesis of diabetes and more recently in mitochondrial alterations in skeletal muscle of diabetic mice. However, so far the exact sources of ROS in skeletal muscle have remained elusive. Aiming at better understanding the causes of mitochondrial alterations in diabetic muscle, we designed this study to characterize the sites of ROS production in skeletal muscle of streptozotocin (STZ)-induced diabetic mice. Hyperglycemic STZ mice showed increased markers of systemic and muscular oxidative stress, as evidenced by increased circulating \( \text{H}_2\text{O}_2 \) and muscle carbonylated protein levels. Interestingly, insulin treatment reduced hyperglycemia and improved systemic and muscular oxidative stress in STZ mice. We demonstrated that increased oxidative stress in muscle of STZ mice is associated with an increase of xanthine oxidase (XO) expression and activity and is mediated by an induction of \( \text{H}_2\text{O}_2 \) production by both mitochondria and XO. Finally, treatment of STZ mice, as well as high-fat and high-sucrose diet-fed mice, with oxypurinol reduced markers of systemic and muscular oxidative stress and prevented structural and functional mitochondrial alterations, confirming the in vivo relevance of XO in ROS production in diabetic mice. These data indicate that mitochondria and XO are the major sources of hyperglycemia-induced ROS production in skeletal muscle that the inhibition of XO reduces oxidative stress and improves mitochondrial alterations in diabetic muscle.

reactive oxygen species; mitochondria; diabetes; oxypurinol

ALTERED LIPID METABOLISM and mitochondrial dysfunction in skeletal muscle have been associated with insulin resistance and type 2 diabetes (13, 28). These alterations in mitochondria structure, density, and/or function (23, 34, 36) have led to the speculation that a decrease in the capacity to oxidize fat due to acquired or inherited mitochondrial defect may be the underlying cause of lipid accumulation and thus a consequence of insulin resistance (24). However, we (5) and others (14, 37, 42, 45) have challenged this hypothesis, and we demonstrated recently that mitochondrial alterations are not a causal event in the development of diet-induced insulin resistance but rather a consequence of oxidative stress in skeletal muscle of diabetic mice (5). Consequently, a better understanding of the exact sites of reactive oxygen species (ROS) generation in skeletal muscle is important to improve mitochondrial alterations and reduce lipotoxicity.

Oxidative stress has also been implicated in the pathogenesis of insulin resistance and diabetes based in part on the role of ROS in micro- and macrovascular complications associated with hyperglycemia in late-stage diabetes (38). Interestingly, ROS production is not confined to vascular cells, and several data also suggest that ROS may be produced in many tissues and particularly in insulin-sensitive tissues (12, 41). Among them, skeletal muscle is now recognized as a potential source of both ROS and reactive nitrogen species (RNS) under various physiological and pathological conditions (16). Type 1 and type 2 diabetes-associated hyperglycemia are pathological situations in which increased oxidative stress could affect the function of skeletal muscle (29). Furthermore, we have shown previously that acute hyperglycemia induces a rapid downregulation of a number of genes in skeletal muscle of healthy subjects likely via a mechanism involving oxidative stress (32). However, mechanisms responsible for the glucose-induced oxidative stress in skeletal muscle remain to be elucidated.

Several cellular sources could be involved in the production of ROS into muscle, including complexes I and III of the electron transport chain of mitochondria (3, 27), membrane-associated NADPH oxidase (10, 18), and xanthine oxidase (XO) (1). In addition, nitric oxide (NO) can also be generated by skeletal muscle, since both neuronal (type I) and endothelial (type III) isoforms of NO synthase (NOS) are expressed in this tissue (2). It has been demonstrated that NO is able to react with superoxides to generate peroxynitrite, suggesting possible interactions between ROS and RNS formed in skeletal muscle, leading to their reduced bioavailability and potentially altered cellular responses (4). In parallel, skeletal muscle possesses a well-developed system to regulate ROS and prevent potentially deleterious effects. These protection systems include both mitochondrial and cytosolic isoforms of superoxide dismutase (MnSOD and CuZnSOD, respectively), catalase and glutathione peroxidase (GPX) enzymes, and a number of direct scavengers of ROS. However, the relative importance of the production and protective pathways has not been established in skeletal muscle in diabetic states. Elucidation of this issue could help to prevent oxidative stress and associated muscle damages in this disease.

Thus the goal of our study was to investigate the pathways involved in hyperglycemia-induced ROS generation in skeletal

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muscle of diabetic mice. We have chosen to study the streptozotocin (STZ)-induced diabetic mice because we have demonstrated previously that oxidative stress induced similar mitochondrial alterations in both type 1 and type 2 diabetic mice (5). The identification of hyperglycemia-induced ROS production systems in skeletal muscle of diabetic STZ mice was performed 1) by measuring the expression and/or enzyme activity of genes related to ROS production and protection and their regulation by insulin and antioxidant treatments in skeletal muscle and 2) by measuring directly H₂O₂ production on both isolated mitochondria and total muscle lysates. Altogether, our findings indicate that activation of mostly mitochondria and XO account for hyperglycemia-induced production of ROS in diabetic muscle and demonstrate for the first time a beneficial effect of oxypurinol, a XO inhibitor, on mitochondrial alterations in skeletal muscle of both type 1 and type 2 diabetic mice.

MATERIALS AND METHODS

Animals

Animals were purchased from Harlan (Gamnat, France) and housed in a room under controlled temperature and humidity and had free access to water and chow. All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals from the National Ethics Committee on Animal Experimentation, Decree No. 2005-264, and the Ethics Committee of Rhône-Apes Region (protocol no. 164) approved the protocols.

Experiment 1. Ten-week-old male C57BL/6 mice (n = 32) were given daily (at 9 AM) an intraperitoneal (ip) dose of STZ dissolved in sodium citrate buffer (100 mg/kg body wt; Sigma), for 3 consecutive days. The control group (Co; n = 16) was injected with sodium citrate buffer. Glucose levels were monitored daily, and when STZ mice achieved fed glucose levels >500 mg/dl for 3 consecutive days (day 11), they were separated into two groups (n = 16/group). One group was injected with saline solution, and the second was treated with insulin (3 mU; Insulatard) twice daily at 8-h intervals. Twenty-four hours after the first injection of insulin, all animals were euthanized, blood was collected, and gastrocnemius muscles were removed and frozen in liquid nitrogen.

Experiment 2. Another group of Co (n = 10) and STZ mice (n = 30) were made as described above. On day 8, STZ mice were separated into three groups (n = 10/group). One group was untreated, the second one was treated with the general antioxidant N-acetyl-cysteine (NAC; 10 g/l in the drinking water), and the third group was treated with oxypurinol (OXY; an inhibitor of XO. 1 mM in the drinking water) until day 12. The concentration of oxypurinol was previously shown to inhibit XO (35). Fresh solutions were made every 48 h. On day 12, all mice were euthanized, blood was collected, and gastrocnemius muscles were removed and frozen in liquid nitrogen.

Experiment 3. Male C57BL/6 mice at 4 wk were fed a standard diet (SD; 57% carbohydrate, 5% fat, 18% protein; Harlan) or a pellet diet (HFHSD; 36% fat, 35% carbohydrate (50% sucrose), and 19.8% protein, TD99249; Harlan) for 16 consecutive weeks. One group of HFHSD mice treated with oxypurinol (1 mM in the drinking water) during all of the feeding period. During the last week of protocol, ip glucose and insulin tolerance tests (IPGTT and IPITT, respectively) were performed on 8-h-fasted mice. Mice were injected ip with 2 mg/g body wt glucose or 0.75 mU/g body wt insulin. Blood was taken by tail puncture immediately before and at different times after injection for measurements of plasma glucose. At the end of the protocol, mice were euthanized, blood was collected in the fed state, and gastrocnemius muscles were fixed for electron microscopy analysis.

Measurement of Metabolites and Hormones

Blood glucose levels were measured using a glucometer (Roche Diagnostics, Meylan, France). Serum levels of insulin were determined using the murine ELISA kits (Linco Research). Plasma H₂O₂ levels were measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen, Cergy Pontoise, France). Specificity of the signal for H₂O₂ was confirmed by treating plasma with polyethylene glycol catalase (100 U/ml).

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted with the Trizol Reagent (Invitrogen). The level of target mRNAs were measured by RT, followed by real-time PCR, using a LightCycler (Roche Diagnostics). Primer sequence and RT-quantitative PCR conditions are available upon request. We measured hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA as a reference gene so that the results are expressed as a ratio referred to the expression of HPRT.

Protein Carbonylation

The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (Hampshire, UK). The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenyl (DNP)-hydrazone by reaction with 2,4-dinitrophenylhydrazine, following the manufacturer’s instructions. After the derivatization of the protein sample (20 µg), one-dimensional electrophoresis was carried out on a 10% SDS-PAGE gel. Proteins were transferred to polyvinylidene difluoride membranes. After incubation with anti-DNP antibody, the blot was developed using a chemiluminescence detection system. After transfer, the gels were stained with Coomassie blue to verify protein loading. These gels were scanned, and the intensity of each lane allowed the normalization of the densitometry analysis of carbonylated proteins.

Measurement of Enzyme Activities

XO activity was measured using the Amplex Red Xanthine Oxidase assay kit from Molecular Probes (MP22182; Molecular Probes) according to the instructions of manufacturer. NOS activity was measured using the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase from Euromedex (NB78) according to the instructions of manufacturer. NADPH oxidase activity was measured according to the protocol of Iverson et al. (15a). Briefly, 200 µg of skeletal muscle proteins was incubated for 60 min at 37°C in a 100-µl volume containing 0.1 M potassium phosphate buffer, pH 5.5, 2 mM potassium cyanide, and 200 µM NADPH. The incubation was stopped by the addition of 0.4 N HClO₄, and proteins were removed by centrifugation. Forty microliters of the resulting supernatant was added to 60 µl of 10 N NaOH and allowed to incubate for 1 h at room temperature. One-hundred microliters of water was added to each sample, and fluorescence was measured in a fluorimeter equipped with a 355-nm primary filter and a 460-nm secondary filter.

Muscle Lysates and Isolation of Muscle Mitochondria

Gastrocnemius and quadriceps muscles were quickly removed and placed in ice-cold lysis medium (100 mM sucrose, 50 mM KCl, 5 mM EGTA, and 50 mM Tris–HCl, pH = 7.4). Muscles were cleaned of extracellular fat and connective tissue, minced with scissors, and used either for muscle lysates (right limb) or for isolation of mitochondria (left limb). For lysates, muscle was homogenized in 1.5 ml of lysis medium and centrifuged at 800 g for 10 min. For isolation of mitochondria, muscle was cut into small pieces in isolation buffer (210 mM mannitol, 70 mM saccharose, 50 mM Tris, 10 mM EDTA, and 0.5% BSA, pH = 7.4). Then, it was digested for 15 min with trypsin under agitation and washed twice with the isolation buffer. After each wash, the tissue was centrifuged for 2 min at 70 g. The
tissue was homogenized with a conical glass grinder (VWR International) in isolation buffer. The homogenate was centrifuged for 10 min at 820 g. Then the supernatant was centrifuged for 20 min at 6,800 g. The pellet was resuspended in suspension buffer (225 mM mannitol, 75 mM saccharose, 10 mM Tris, and 0.1 mM EDTA, pH = 7.4) and centrifuged for 10 min at 820 g. The mitochondria were then pelleted by centrifuging the supernatant for 20 min at 6,800 g and resuspended in 100 μl of the same buffer. Protein quantification of muscle lysates and isolated mitochondria was measured by the Biuret method, with BSA as standard.

Mitochondrial H2O2 Production

Generation of mitochondrial H2O2 was determined on an SFM25 computer-controlled Kontron fluorometer by measuring the linear increase in fluorescence (excitation at 560 nm, emission at 584 nm) due to enzymatic oxidation of Amplex Red by H2O2 in the presence of horseradish peroxidase. Mitochondria (0.1 mg/ml) were incubated at 30°C in a buffer containing 125 mM KCl, 20 mM Tris·HCl, 1 mM EGTA, 0.15% fat-free BSA (pH 7.2), 6 U/ml horseradish peroxidase, and 1 μM Amplex Red. The reaction was started by addition of succinate (5 mM). Mitochondrial H2O2 was measured in basal conditions, and 2 M rotenone and 2 M antimycin A were sequentially added to determine the maximum rate of H2O2 production of complexes I and I + III of the respiratory chain, respectively. To verify the specificity of the Amplex Red reaction with H2O2, catalase (100 U/ml) was also added to mitochondria.

XO-Mediated H2O2 Production

Generation of H2O2 by the XO was determined in the same conditions as in mitochondrial H2O2 production. Muscle lysates (100 μl) were incubated at 30°C in a buffer containing 125 mM KCl, 20 mM Tris·HCl, 1 mM EGTA, 0.15% fat-free BSA (pH 7.2), 6 U/ml horseradish peroxidase, and 1 μM Amplex Red. The reaction was started by addition of succinate (5 mM). Mitochondrial H2O2 was measured in basal conditions, and 2 M rotenone and 2 M antimycin A were sequentially added to determine the maximum rate of H2O2 production of complexes I and I + III of the respiratory chain, respectively. To verify the specificity of the Amplex Red reaction with H2O2, catalase (100 U/ml) was also added to mitochondria.

Measurement of ATP Synthesis

ATP synthesis by isolated mitochondria from skeletal muscle was measured according to the protocol of Vives-Bauza et al. (44). Briefly, freshly isolated mitochondria were diluted in 160 μl of a specific buffer (150 mM KCl, 25 mM Tris·HCl, 2 mM EDTA, 0.1% BSA, 10 mM potassium phosphate, pH 7.4) containing P1,P5-di(adenosine) pentaphosphate (0.15 mM). Luciferin, luciferase, ADP, and either malate plus pyruvate (to 1 mM) or succinate (to 5 mM) plus 2 μg/ml rotenone were added to the mitochondria suspension. Measurements of ATP synthesis were performed using a luminometer, which allows recordings in the kinetic mode. To obtain baseline fluorescence corresponding to nonmitochondrial ATP production, one replicate tube for each sample was prepared containing the components described above plus 1 μg/ml oligomycin.

Transmission Electron Microscopy

Transmission electron microscopy on skeletal muscle was performed as described previously (5). Briefly, gastrocnemius muscle was cut into small pieces and fixed in 2% glutaraldehyde for 2 h at 4°C, postfixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated, and embedded in Epon at either a longitudinal or transverse orientation. The tissue was then cut using a RMC/MTX ultramicrotome (Electron Microscopy Sciences) at 70–90 nm for transmission electron microscopy. The tissue was then cut using a RMC/MTX ultramicrotome (Electron Microscopy Sciences) at 70–90 nm for transmission electron microscopy. The tissue was then cut using a RMC/MTX ultramicrotome (Electron Microscopy Sciences) at 70–90 nm for transmission electron microscopy.

RESULTS

Increased Oxidative Stress in Skeletal Muscle of STZ Mice

To determine whether oxidative stress takes place in skeletal muscle in the diabetic state, we analyzed STZ-induced diabetic mice as a model of hyperglycemia-associated oxidative stress without obesity. In addition, we examined the effect of acute insulin treatment (to reduce hyperglycemia) on systemic and muscle oxidative stress in STZ mice. As shown in Table 1, STZ mice exhibited decreased body weight (20%, P < 0.001), severe hyperglycemia (225%, P < 0.001), hypoinsulinemia (77.5%, P < 0.001), and increased plasma H2O2 levels (121%, P < 0.001), demonstrating type 1 diabetes-associated systemic oxidative stress. The specificity of H2O2 measurement in plasma was validated using catalase, which inhibits the Amplex Red signal (data not shown). Insulin treatment of STZ mice increased body weight (12%, P < 0.01), decreased glucose levels (19%, P < 0.001), and significantly reduced plasma H2O2 levels (~38%, P < 0.05) compared with untreated STZ mice.

As a marker of muscle oxidative stress, we measured total protein carbonylation levels in gastrocnemius muscle of control mice, untreated STZ mice, and insulin-treated STZ mice. As illustrated in Fig. 1A, protein carbonylation is markedly increased in skeletal muscle of STZ mice compared with control mice, suggesting an oxidative stress in muscle of diabetic mice. Insulin treatment reduced protein carbonylation in skeletal muscle of STZ mice compared with untreated STZ mice, suggesting a reduction of ROS production and/or an increase of antioxidant defenses in muscle of insulin-treated STZ mice.

Next, we evaluated the activity of ROS and their production sites. We first measured changes in mRNA levels of oxidant and antioxidant enzymes in skeletal muscle of mice. Concerning prooxidant enzymes, we measured the mRNA levels of 1) uncoupling protein (UCP)2 and -3 as markers of mitochondria-mediated ROS production, 2) gp91, p22, p40, p47, and p67 subunits of NADPH oxidase, 3) XO, and 4) the endothelial (NOS3) and neuronal (NOS1) NO synthases as an index of RNS production. We found that UCP2, UCP3, and XO mRNA levels were significantly increased in skeletal muscle of STZ mice.

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<th>Table 1. Characteristics of mice in experiment 1</th>
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<td>Body weight, g</td>
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<td>Glucose, mg/dl</td>
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<td>Insulin, ng/ml</td>
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<td>H2O2, μM</td>
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Data represent means ± SE of 16 mice/group. Co, control mice; STZ, untreated streptozotocin-treated mice; STZ + INS, insulin-treated STZ mice. Body weight, glycemia, insulinemia, and plasma H2O2 concentrations of Co, STZ, and STZ + INS mice. #P < 0.001 vs. Co mice; *P < 0.05; **P < 0.01 vs. STZ mice.
mice compared with control mice, whereas none of the subunits of NADPH oxidase, as well as NOS1 and NOS3, were altered (Fig. 1B), suggesting that ROS production in skeletal muscle of diabetic mice may implicate mainly mitochondria and XO. Insulin treatment restored the mRNA levels of UCP2 \((P < 0.05)\) and UCP3 \((P = 0.06)\), whereas expression of XO was not affected, compared with untreated STZ mice (Fig. 1B). In addition, insulin further reduced SOD2 expression in skeletal muscle of STZ mice (Fig. 1B).

Then, we measured enzyme activities of prooxidant enzymes. As shown in Fig. 2A, XO activity was significantly increased in skeletal muscle of STZ mice compared with Co mice \((35\%, \ P < 0.01)\), whereas neither NADPH oxidase nor NOS activities were modified. Insulin treatment did not modify any of these activities (Fig. 2A).

Altogether, these results indicate that there is an increase of oxidative stress markers in skeletal muscle of STZ-induced diabetic mice compared with control mice, whereas none of the subunits of NADPH oxidase, as well as NOS1 and NOS3, were altered (Fig. 1B), suggesting that ROS production in skeletal muscle of diabetic mice may implicate mainly mitochondria and XO. Insulin treatment restored the mRNA levels of UCP2 \((P < 0.05)\) and UCP3 \((P = 0.06)\), whereas expression of XO was not affected, compared with untreated STZ mice (Fig. 1B).
diabetic mice, which could be mediated by either an increase of mitochondria and XO-mediated ROS production and/or a reduction of some antioxidant enzymes (SOD2).

**Increased Mitochondria and XO-Mediated H₂O₂ Production in STZ Mice**

To confirm the implication of mitochondria in hyperglycemia-induced ROS production, we measured succinate-induced H₂O₂ production by isolated muscle mitochondria of control, untreated, and insulin-treated STZ mice (n = 6) in nonphosphorylated conditions (33). We have validated the specificity for H₂O₂ measurement by demonstrating that the addition of catalase inhibited the Amplex Red signal (data not shown). As shown in Fig. 2B, succinate (providing FADH₂ to complex II of electron transfer chain) induced H₂O₂ production by control mitochondria (+25-fold, P < 0.01), and this production is drastically inhibited by rotenone (−84%, P < 0.01), suggesting that it is due mainly to an enhanced ROS generation through a reverse electron flow at the level of complex I of the respiratory chain. Then, addition of antimycin A modestly induced H₂O₂ production, compared with that measured with rotenone (Fig. 2B), indicating a weak implication of complex III in H₂O₂ production energized with succinate. Interestingly, the rate of H₂O₂ production by STZ mice-derived mitochondria was significantly higher than from control mice (+164%, P < 0.05; Fig. 2B), indicating an increase of mitochondrial H₂O₂ production in skeletal muscle of STZ mice. Insulin treatment of the mice tended to reduce mitochondrial H₂O₂ production energized with succinate (−65%, P = 0.07; Fig. 2B).

To confirm the implication of XO in hyperglycemia-induced ROS production, we accessed xanthine-stimulated H₂O₂ production on total muscle lysates of control, untreated, and insulin-treated STZ mice (n = 6). As shown in Fig. 2C, xanthine induced H₂O₂ production by control muscle lysates (1.6 fold, P < 0.05), and this production is totally inhibited by addition of allopurinol (P < 0.01), confirming the involvement of XO in the measured H₂O₂ production. Interestingly, the rate of xanthine-induced H₂O₂ production by STZ muscle lysates was significantly higher than in muscle lysates from control mice (+45%, P < 0.05; Fig. 2C), suggesting that XO participates in the increase of ROS production in skeletal muscle of STZ mice. Once again, insulin treatment tended to reduce xanthine-induced H₂O₂ production compared with untreated STZ mice (−87%, P = 0.06; Fig. 2C).

Taken together, our results indicate that both complex I of mitochondria and XO contribute to increased H₂O₂ production in skeletal muscle of hyperglycemic STZ mice.

**Inhibition of XO by Oxypurinol Treatment Reduces Oxidative Stress in STZ Mice**

We then sought to determine whether inhibition of XO could improve oxidative stress in skeletal muscle of diabetic mice in vivo. STZ mice were treated with oxypurinol (1 mM) for 7 consecutive days, and the consequences on systemic and muscle oxidative stress markers were measured. In parallel, another group was treated with a general antioxidant, NAC (10 g/l). The metabolic data of the animals were presented in Table 2. Oxypurinol treatment reduced circulating H₂O₂ (−36%, P < 0.01), without changes in plasma glucose and insulin levels, compared with untreated STZ mice (Table 2). NAC-treated STZ mice showed an improvement in glycemia (21%, P < 0.05), without significant change of circulating insulin and H₂O₂ levels, compared with untreated STZ mice (Table 2). Concerning muscle oxidative stress, treatment of STZ mice with oxypurinol reduced muscle protein carbonylation levels (−30%, P < 0.05) compared with untreated STZ mice (Fig. 3A). Similarly, NAC treatment decreased protein

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<th>Table 2. Characteristics of mice in experiment 2</th>
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<td>Co</td>
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<tr>
<td>Body weight, g</td>
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<td>Glucose, mg/dl</td>
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<td>Insulin, ng/ml</td>
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<td>H₂O₂, μM</td>
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Data represent means ± SE of 10 mice/group. NAC, N-acetyl cysteine; OXY, oxypurinol. Body weight, glycemia, insulinemia, and plasma H₂O₂ concentrations of Co, STZ, and NAC (STZ + NAC)- or OXY (STZ + OXY)-treated STZ mice. #P < 0.001 vs. Co mice; *P < 0.01 vs. STZ mice.
carbonylation levels compared with untreated STZ mice (Fig. 4A). In addition, mRNA levels of almost all oxidant and antioxidant enzymes were poorly regulated by both antioxidants (Figs. 3B and 4B). We only observed a decrease in mRNA levels of p22 and p40 subunits of NADPH oxidase and of GSR in oxypurinol-treated STZ compared with untreated STZ mice (Fig. 3B). Altogether, these results show that inhibition of XO by oxypurinol is sufficient to improve circulating and muscular oxidative stress markers in STZ-induced diabetic mice.

**Treatment of STZ Mice With Oxypurinol Improved Mitochondria Structure and Function**

We have demonstrated previously that oxidative stress in hyperglycemic STZ mice is associated with altered mitochondria structure and function in skeletal muscle and that NAC treatment restored mitochondrial alterations (5). Since we demonstrated here that XO is implicated in hyperglycemia-associated muscle oxidative stress, we investigated whether oxypurinol treatment could also have a beneficial effect on mitochondria structure and density. First, we confirmed that oxypurinol specifically inhibited XO activity in skeletal muscle of STZ mice, whereas NADPH and NOS activities were not modified by oxypurinol treatment (Fig. 5). Then, we analyzed by electron microscopy the structure and density of mitochondria in muscle of Co, untreated STZ, and oxypurinol-treated STZ mice. As shown in Fig. 6, we observed both a reduction of mitochondria density and important alterations of both subsarcolemmal and intermyofibrillar mitochondria structure in skeletal muscle of STZ mice compared with Co mice. These alterations included mitochondria swelling, an increased number of disarrayed cristae, and a reduced electron density of the matrix. Interestingly, oxypurinol treatment improved these structural alterations, since we observed an increase in the number of mitochondria per section and a restoration of both cristae structure and electron density of the matrix (Fig. 6), suggesting a role of XO in mitochondrial alterations in muscle of STZ mice.

We then measured ATP production by isolated mitochondria to access mitochondria function in muscle of untreated and oxypurinol-treated STZ mice. As shown in Fig. 7, ATP production mediated by succinate in the presence of rotenone is reduced in muscle of STZ mice compared with Co mice (−30%, P < 0.05), whereas ATP production mediated by complex I substrates (glutamate + malate) was not modified. Interestingly, oxypurinol treatment restored succinate-induced ATP production in muscle of STZ mice (Fig. 7).

Altogether, these data indicate that reduction of XO activity improves mitochondria structure and function in muscle of STZ mice.

**Effect of Oxypurinol Treatment in Diet-Induced Diabetic Mice**

To determine whether oxypurinol treatment could also be beneficial for mitochondrial alterations in a model of type 2 diabetes, we investigated mitochondria structure in SD-fed mice, HFHSD-fed mice, and oxypurinol-treated HFHSD mice.
for 16 wk. Metabolic characteristics of these mice were shown in Table 3. HFHSD feeding clearly induced an obese and diabetic phenotype compared with SD mice, since HFHSD mice weighed 18 g more, were hyperglycemic and hyperinsulinemic (Table 3), and showed altered response to glucose and insulin tolerance tests (Table 3). Treatment of HFHSD mice with oxypurinol did not modify body weight, glycemia, insulinemia, and response to glucose and insulin tolerance tests. Nevertheless, oxypurinol treatment reduced plasma H2O2 levels in HFHSD mice compared with untreated mice (Table 3). As described previously (5), HFHSD mice showed altered mitochondria structure com-

![Figure 6](http://ajpendo.physiology.org/)

Fig. 6. OXY treatment restored alterations of mitochondria structure in skeletal muscle of STZ mice. Co or STZ-induced diabetic mice were studied 12 days after the 1st injection of sodium citrate buffer or STZ, and OXY was added to drinking water of STZ mice during the last 5 days of the protocol. Transmission electron microscopy images (original magnification ×15,000 or ×40,000) of a representative muscle fiber (left) and of a zoom on subsarcolemmal (middle) and intermyofibrillar (right) mitochondria from the gastrocnemius muscle of Co, STZ, and OXY-treated STZ mice. Arrows point to mitochondria swelling, increased disarrayed cristae, and reduced electron density of the matrix.

![Figure 7](http://ajpendo.physiology.org/)

Fig. 7. OXY treatment improved mitochondria function in skeletal muscle of STZ mice. Co or STZ-induced diabetic mice were studied 12 days after the 1st injection of sodium citrate buffer or STZ, and OXY was added to drinking water of STZ mice during the last 5 days of the protocol. ATP synthesis was measured on isolated mitochondria from gastrocnemius muscle of Co, STZ, and OXY-treated STZ mice. Values are means ± SE (n = 5). *P < 0.05 vs. Co; #P < 0.05 vs. STZ.

![Table 3](http://ajpendo.physiology.org/)

**Table 3. Characteristics of mice in experiment 3**

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<th>SD</th>
<th>HFHSD</th>
<th>HFHSD + OXY</th>
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<td>Body weight, g</td>
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<td>44.7 ± 1.1 *</td>
<td>45.5 ± 1.4</td>
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<td>Glucose, mg/dl</td>
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<td>192.3 ± 5.7 *</td>
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<td>Insulin, ng/ml</td>
<td>0.5 ± 0.05</td>
<td>3.7 ± 0.7 *</td>
<td>4.2 ± 0.7</td>
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<td>AUC for IPGTT (AU)</td>
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<td>1.45 ± 0.35</td>
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<td>AUC for IPITT (AU)</td>
<td>1 ± 0.3</td>
<td>3.42 ± 0.25 *</td>
<td>3.1 ± 0.15</td>
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<tr>
<td>H2O2, µM</td>
<td>28 ± 4</td>
<td>43.5 ± 4 *</td>
<td>30.1 ± 6 #</td>
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Data represent means ± SE of 10 mice/group. SD, standard diet; HFHSD, untreated high-fat and high-sucrose-fed mice; HFHSD + OXY, oxypurinol-treated HFHSD mice; AUC, area under the curve; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; AU, arbitrary units. Body weight, glycemia, insulinemia, AUC for both IPGTT and IPITT, and plasma H2O2 concentrations of SD-fed, HFHSD-fed, and HFHSD + OXY mice. *P < 0.01 vs. SD mice; #P < 0.05 vs. untreated HFHSD mice.
pared with SD mice, as demonstrated by the reduction and the swelling of both subsarcolemmal and intermyofibrillar mitochondria associated with the increased number of disarrayed cristae and the reduced electron density of the matrix (Fig. 8). Interestingly, oxypurinol treatment improved these alterations in muscle of HFHSD mice (Fig. 8), confirming the preventive effect of blocking XO-mediated ROS production on mitochondria structure in diet-induced diabetic mice.

DISCUSSION

Hyperglycemia-induced systemic oxidative stress is a classic feature of diabetes. In addition, we have demonstrated recently that oxidative stress is one of the culprits of mitochondrial dysfunction in skeletal muscle of both STZ- and diet-induced diabetic mice (5). However, whether muscle ROS generation is really increased and the intrasubcellular sources of production in diabetic states are poorly known. The purpose of our study was to investigate this issue in STZ-induced diabetic mice, which share similar oxidative stress-induced mitochondrial alterations to diet-induced diabetic mice, to identify the main sources of hyperglycemia-induced ROS production in skeletal muscle. Then, specific antioxidant strategies were tested in both models to study the repercussions on mitochondrial alterations in skeletal muscle of diabetic mice.

In the present study, we used STZ-induced diabetic mice as a classical model of hyperglycemia-induced oxidative stress (39), and we treated these mice with insulin, NAC, or oxypurinol to prevent this process. STZ is used extensively to induce pancreatic β-cell death and ultimately diabetes mellitus in animal models. Whereas the direct effects of STZ on muscle cannot be excluded (21), the comparison of phenotype between STZ and STZ plus insulin should help to delineate the effects of STZ from the effects of hypoinsulinemia/hyperglycemia. STZ-treated mice showed hyperglycemia, hypoinsulinemia, and increased systemic and muscular oxidative stress compared with control mice. Interestingly, insulin treatment decreased both plasma glucose and H2O2 levels and markedly reduced intramuscular protein carbonylation, suggesting that hyperglycemia (and not STZ treatment per se) could be directly causal in muscle ROS production. Nevertheless, we cannot exclude that hypoinsulinemia also contributed to increased oxidative stress in STZ mice and that insulin treatment per se participated in reduced oxidative stress in muscle of insulin-treated STZ mice. Indeed, the reduction of hyperglycemia by insulin treatment is modest in STZ mice, whereas insulin-induced improvement of both circulating and muscle oxidative stress is more marked. We believe that the apparent mild reduction in glycemia obtained after ip insulin administration
in STZ mice reflected transitory action of insulin under our experimental procedure (2 administrations of insulin, 24 and 15 h before euthanization). Despite the rebound of glycemia after insulin infusion, muscle protein carbonylation and, more importantly, structure and density of mitochondria were restored (5). These results suggest that, in addition to the improvement of glycemia, specific effects of insulin should be taken into account to explain the reduction of oxidative stress and mitochondria rescue. These observations are in agreement with recent data demonstrating a beneficial effect of insulin on lipopolysaccharide-induced oxidative stress independent of any change in glucose concentrations (8). Whereas the molecular mechanisms by which insulin can reduce oxidative stress require further investigations, we can already conclude from our study that it does not involve a regulation of prooxidant enzyme activities since they are not regulated by insulin treatment in muscle of STZ mice.

Two important points arising from our study are that skeletal muscle of diabetic mice showed increased ROS production and that this production is mediated by both the complex I of mitochondria and XO. This was supported by several independent experiments. First, data on the expression of genes implicated in ROS production and protection suggest that oxidative stress in STZ mice is related more to an increase in ROS production than a decrease in antioxidant defenses. Indeed, the mRNA levels of antioxidant enzymes are deregulated weakly in skeletal muscle of STZ mice, except for SOD2, which showed reduced expression. The others are either induced (GSR or catalase) or not regulated (Gpx, Prdx3, and Prdx5). In contrast, mRNA levels of UCP2, UCP3, and XO were increased significantly in skeletal muscle of STZ mice, suggesting contribution of mitochondria and XO in ROS production. This was further supported by the increase of XO activity, without modification of both NADP and NOS activities, in skeletal muscle of STZ mice. The final proof was given by the direct measurements of H$_2$O$_2$ production in skeletal muscle of STZ mice. To our knowledge, there is sparse work directly measuring ROS production in diabetic skeletal muscle, and generally these measurements were performed on isolated mitochondria. Here, we measured H$_2$O$_2$ production by mitochondria, but we also tried to investigate XO-induced H$_2$O$_2$ production by measuring xanthine-stimulated H$_2$O$_2$ production on total muscle lysates. The specificity of XO-related ROS production was confirmed by inhibition with allopurinol, an inhibitor of XO. We observed both an increase of H$_2$O$_2$ production by isolated mitochondria and an increase of xanthine-induced H$_2$O$_2$ production in skeletal muscle of STZ mice compared with control mice, confirming the implication of both pathways in ROS production in skeletal muscle of diabetic mice. Consequently, the increase of both XO expression and activity participate in increased ROS production in skeletal muscle of STZ mice.

It is not surprising that mitochondria was implicated in hyperglycemia-induced ROS production, since mitochondria were known to be the major site of ROS production in many tissues and in a wide range of pathological conditions (19). Most importantly, our results showed that XO, a key enzyme of purine metabolism, is also a significant cellular source of ROS production in muscle of diabetic mice. Normally, XO activity is low in skeletal muscles (6). However, ROS production by XO is favored by an accumulation of intracellular ADP (20). Consequently, mitochondrial dysfunction observed in skeletal muscle of STZ mice (5) may lead to an imbalance of ATP/ADP ratio in the cytosol and an associated increase of XO activity. XO was reported to localize in capillary endothelial cells (17) and in the sarcolemma (15). Consequently, we cannot exclude that part of XO activity arises from endothelial cells. In addition, it is difficult to evaluate whether mitochondria and XO have additive effects on ROS production in vivo since the structure of mitochondria is altered in muscle of STZ. Indeed, we have observed by electron microscopy that purified mitochondria of both control and STZ mice were equally intact (data not shown), suggesting that the most damaged mitochondria were lost during differential centrifugation. Since it is not known whether or not the most damaged mitochondria can produce superoxide, it is not possible to conclude on the relative implication of both sources of ROS in vivo. It is likely that both are implicated but maybe with different kinetics during the development of STZ-induced diabetes. The implication of XO in ROS production by skeletal muscle of diabetic mice is supported by other studies demonstrating either that type 1 diabetes is associated with XO-mediated ROS production in other tissues (9, 30) or that skeletal muscle is able to generate ROS through XO in other physiopathological situations (22, 26, 43).

To assess whether XO could be a potential target to protect against damages associated with oxidative stress in diabetes, we have treated STZ with oxypurinol and measured consequences on systemic and muscular oxidative stress markers. Oxypurinol treatment reduced both circulating H$_2$O$_2$ concentrations and muscle protein carbonylation levels, confirming the implication of XO in hyperglycemia-induced ROS production in skeletal muscle of STZ mice. Interestingly, oxypurinol treatment restored structural and functional alterations of mitochondria in skeletal muscle of STZ mice, indicating that XO inhibition could be useful to prevent muscle damage. The expression of XO is not modified by oxypurinol treatment, indicating that the action of the drug involved only inhibition of XO activity. The inhibition of XO by oxypurinol probably led to a decrease in cytosolic ROS production, contributing to the observed reduced protein oxidation. Nevertheless, we cannot exclude that the decrease of circulating H$_2$O$_2$ participated in the reduced protein carbonylation in skeletal muscle of STZ mice. This systemic effect of oxypurinol suggests that XO is also a major source of ROS in vascular cells, confirming previous data (31). Since ROS was implicated in hyperglycemia-induced diabetic complications (7), it is tempting to hypothesize that inhibition of XO could be beneficial toward improving micro- and macrovascular complications associated with diabetes. Interestingly, we confirmed the beneficial effect of oxypurinol treatment on mitochondrial alterations in skeletal muscle of diet-induced diabetic mice, a model of type 2 diabetes. This preventive effect was not accompanied by change in either plasma glucose or insulin levels or in response to IPGTT and IPITT, suggesting that improvement of mitochondria integrity by oxypurinol is not sufficient to improve insulin sensitivity in HFD mice. Since diet-induced diabetic mice have a more complex metabolic phenotype, with an increase of both circulating and ectopic lipid levels and of inflammation, it is likely that other prooxidant enzymes (11) and/or other stress-activated pathways are always activated and participate in the maintenance of insulin resistance. Nevertheless, this result is
an additional argument claiming that mitochondrial dysfunction is not a causal event of insulin resistance (14, 37, 42, 45). Nevertheless, by promoting intramuscular lipid accumulation, mitochondrial dysfunction could participate in the maintenance or in the exacerbation of impaired insulin sensitivity. Consequently, our data suggest that the inhibition of XO should be a useful strategy to improve mitochondrial alterations, to stimulate oxidative capacities, and to reduce lipotoxicity. In agreement, XO activity is induced in type 2 diabetic patients (25). Interestingly, allopurinol is classically used in clinic to treat gout, and patients who are affected by gout show increased circulating uric acid levels and a predisposition to metabolic syndrome (40). Nevertheless, further studies are needed to confirm whether the inhibition of XO may have benefits for diabetic patients.

In summary, the present study demonstrates that there is a hyperglycemia-induced oxidative stress in skeletal muscle of diabetic mice, which results more from an increase of free radical production rates than a reduction of endogenous antioxidant levels. In addition, our data provide in vivo evidence for the contribution of both mitochondria and XO to muscle oxidative stress in states of diabetes. The implication of XO is significant since oxypurinol treatment is able to reduce protein oxidation and improve mitochondrial alterations in both type 1 and type 2 models of diabetic mice. Consequently, XO might be a potential therapeutic target for decreasing muscle damages associated with oxidative stress and more particularly for improving oxidative capacities of muscle in diabetic states.

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DISCLOSURES

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

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