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Enhanced autophagy plays a cardinal role in mitochondrial dysfunction in type 2 diabetic Goto–Kakizaki (GK) rats: ameliorating effects of (−)-epigallocatechin-3-gallate

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Abstract

Oxidative stress and mitochondrial dysfunction are known to play important roles in type 2 diabetes mellitus (T2DM) and insulin resistance. However, the pathology of T2DM remains complicated; in particular, the mechanisms of mitochondrial dysfunction in skeletal muscle and other insulin-sensitive tissues are as yet unclear. In the present study, we investigated the underlying mechanisms of oxidative stress and mitochondrial dysfunction by focusing on mitochondrial dynamics, including mitochondrial biogenesis and autophagy, in skeletal muscle of a nonobese diabetic animal model — the Goto–Kakizaki (GK) rat. The results showed that GK rats exhibited impaired glucose metabolism, increased oxidative stress and decreased mitochondrial function. These dysfunctions were found to be associated with induction of LC3B, Beclin1 and DRP1 (key molecules mediating the autophagy pathway), while they appeared not to affect the mitochondrial biogenesis pathway. In addition, (−)-epigallocatechin-3-gallate (EGCG) was tested as a potential autophagy-targeting nutrient, and we found that EGCG treatment improved glucose tolerance and glucose homeostasis in GK rats, and reduced oxidative stress and mitochondrial dysfunction in skeletal muscle. Amelioration of excessive muscle autophagy in GK rats through the down-regulation of the ROS-ERK/JNK-p53 pathway leads to improvement of glucose metabolism, reduction of oxidative stress and inhibition of mitochondrial loss and dysfunction. These results suggest (a) that hyperglycemia-associated oxidative stress may induce autophagy through up-regulation of the ROS-ERK/JNK-p53 pathway, which may contribute to mitochondrial loss in soleus muscle of diabetic GK rats, and (b) that EGCG may be a potential autophagy regulator useful in treatment of insulin resistance.

Keywords: Type 2 diabetes mellitus; Hyperglycemia; Insulin resistance; Oxidative stress; Mitochondrial dysfunction; Fission; Autophagy

Type 2 diabetes mellitus (T2DM) is becoming an epidemic disease with increasing global prevalence [1]. Patients with T2DM are characterized by hyperglycemia leading to a number of consequential complications such as vascular disease [2]. The underlying pathogenic mechanism of T2DM has long been a research hotspot due to its complexity, and the importance of insulin resistance (IR) during early onset and progression of T2DM is widely accepted [3]. A considerable body of evidence links mitochondrial function to IR [4]. Particularly in skeletal muscle, IR results from mitochondrial dysfunction due to defects in mitochondrial oxidative phosphorylation and reduced mitochondrial density, both of which lead to accumulation of intramyocellular fatty acid metabolites [5–9]. Yet the relationship and "cause-and-effect" between mitochondrial dysfunction and IR remain unclear: while either inherited or aging-related mitochondrial

Abbreviations: AMPK, 5′-AMP-activated protein kinase; DRP1, dynamin-related protein 1; EGCG, (−)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; GK rats, Goto–Kakizaki rats; HO1, heme oxygenase 1; IR, insulin resistance; JNK, c-Jun N-terminal kinase; LC3B, microtubule-associated protein 1 light chain 3B; MAPK, mitogen-activated protein kinases; MDA, malondialdehyde; Mfn1, mitofusin 1; Mfn2, mitofusin 2; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; NQO1, NAD(P)H:quinine oxidoreductase 1; OGTT, oral glucose tolerance test; OPA1, optic atrophy 1 gene protein; PE, phosphatidylethanolamine; PGC1, peroxisome proliferator-activated receptor γ co-activator-1; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, Type 2 diabetes mellitus; VDAC1, voltage-dependent anion-selective channel protein 1.

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dysfunction may give rise to IR [7,8], mitochondrial dysfunction resulting from oxidative stress induced by a high-fat diet appears to be a successor of IR [10].

However, for T2DM patients that exhibit both insulin resistance and mitochondrial dysfunction, mitochondria-targeted therapy seems to be an effective measure. For example, IR is reduced by pharmacological intervention with drugs such as thiazolidinediones or metformin, all of which reduce oxidative stress and induce mitochondrial biogenesis [11–13]. Nevertheless, conventional anti-diabetic drugs carry some inherent drawbacks, for example, by increasing heart failure risk and body weight gain [14]. In the past few years, we have identified a series of mitochondria-targeted antioxidants, metabolites and natural compounds as effective promoters of mitochondrial function, so-called "mitochondrial nutrients" [15,16]. These mitochondria-specific nutrients have fascinating effects on treating T2DM. As shown in a previous publication, the combination of R-α-lipoic acid and acetyl-L-carnitine complementarily mimics the effect of PPARγ/α dual ligands to promote mitochondrial synthesis and adipocyte metabolism in 3T3-L1 adipocytes [17], as well as improve β-cell dysfunction [18]. In two other studies, we reported that a combination of R-α-lipoic acid, acetyl-L-carnitine, nicotinamide and biotin (a) showed antidiabetic effects comparable to pioglitazone without causing weight gain and (b) improved immune dysfunction in diabetic Goto–Kakizaki (GK) rats [19,20].

In one of those studies, we found that mitochondrial DNA copy number and components of the mitochondrial respiratory chain were decreased in GK rats [19]. These decreases may represent the primary hallmarks of mitochondrial dysfunction in GK rats. However, the factors mediating mitochondrial reduction remain unknown. Two microarray-based studies revealed definite contributions of PGC-1 to the mitochondrial loss [21,22]. Yet later research implies that, instead of mitochondrial biogenesis, other mechanisms may be involved [5,23]. Emerging evidence reveals that dysfunctional mitochondria are eliminated by autophagy (or mitochondrial autophagy) [24,25]. In the present study, we investigated whether mitochondrial degradation by autophagy causes the mitochondrial loss in GK rats. In addition, we studied the mitochondria-targeted functions of (−)-epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, whose hypoglycemic effects have been demonstrated both *in vivo* and *in vitro* [26–29]. At a dose of 100 mg/kg/d, EGCG reduced hyperglycemia and improved insulin resistance in GK rats, via preventing ROS-ERK/JNK-p53-mediated mitochondria autophagy. The results broaden our knowledge of EGCG's antidiabetic properties and bolster our proposition that its effects on mitochondria play an important role. Overall, we confirmed that mitochondrial functional improvement shows salutary effects in the context of T2DM in the nonobese diabetic GK rat.

1. Materials and methods

1.1. Animals

Four-week-old male diabetic GK rats together with age-matched male nondiabetic Wistar rats were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animals were housed at 18°C–25°C under 12-h light and dark cycles and allowed access to food and water *ad libitum*. The National Institutes of Health principles of laboratory animal use and care [30] were strictly followed with institutional approval of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

1.2. Reagents

Anti-tubulin was obtained from Sigma (St. Louis, MO, USA); anti-OxPhos complex I 30-kDa subunit was from Invitrogen (Carlsbad, CA, USA); anti-NQO1, anti-HO1, anti-SOD2, anti-VDAC1, anti-PGC1, anti-mtTFA, anti-DRP1, anti-Mfn1, anti-Mfn2, anti-OPA1, anti-JNK, anti-phospho-JNK (Thr 183/Tyr 185), anti-ERK, anti-p53 were from Santa Cruz (Heidelberg, Germany); anti-Beclin1, anti-LC3B, anti-phospho-ERK1/2 (Thr 202/Tyr 204), anti-p-Akt (Ser 473), anti-Akt, anti-p-GSK3β (Ser 9) were from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L), peroxidase-conjugated AffiniPure rabbit anti-mouse IgG (H+L) and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) were from Jackson ImmunoResearch (West Grove, PA, USA). Cell lysis buffer for Western and IP was from Beyotime (Haimen, China), OxyBlot protein oxidation detection kit was from Chemicon (Canada), Pierce ECL Western blotting substrate was from Thermo Scientific (Rockford, IL, USA), BCA protein assay kit was from Thermo Scientific (Rockford, IL, USA), and malondialdehyde (MDA) detection kit was from Jiancheng Biochemical Inc. (Nanjing, China).

1.3. Experimental protocol

Three groups (n=12 in each group) of rats were used: Wistar and GK as control groups, EGCG-treated GK as the experimental group. The experimental group received EGCG (100 mg/kg/d) by gavage. The control groups received the same volume of saline, also administered by gavage. The treatments began 4 weeks postweaning and continued for 3 months. At approximately 18 weeks of age, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and sacrificed for obtaining soleus muscle tissue.

1.4. Oral glucose tolerance test

An oral glucose tolerance test (OGTT, 5g/kg body weight) was performed after starting EGCG administration following a previous GK rat study protocol [31]. All rats were fasted overnight before OGTT. Blood was taken from the retrobulbar vein at 0, 30, 60 and 120 min after oral glucose administration. Plasma glucose concentrations were determined by the glucose oxidase method.

1.5. Western blotting

For protein immunoblot assay, approximately 100 mg muscle was homogenized in ice-cold cell lysis buffer. Protein concentrations were determined using BCA protein assay kit. Soluble lysates (10–15 μg per lane) were subjected to 8%, 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk/TBST for 1–2 h at room temperature. Membranes were incubated with primary antibodies (diluted by 1:5000–1:10,000) for 1–2 h at room temperature. After washing membranes with TBST three times, membranes were incubated with horseradish-peroxidase-conjugated antibody for 1 h at room temperature followed by three washings. Western blots were developed using Pierce ECL Western blotting substrate and quantified by scanning densitometry.

1.6. Protein carbonylation assay

Protein oxidation was detected using OxyBlot Protein Oxidation Detection Kit strictly following the kit protocol. Briefly, protein samples were prepared from soleus muscle. Carbonyl groups were derivatized and subjected to 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and blocked with 1% BSA/TBST for 1 h at room temperature. Membranes were incubated with 1° antibody (diluted 1:150). After washing membranes with TBST three times, membranes were incubated with 2° antibody (diluted 1:300) for 1 h at room temperature followed by three washings. Western blots were developed using Pierce ECL Western blotting substrate and quantified by scanning densitometry. As negative controls, equal amounts of samples were subjected to 10% SDS-PAGE and stained with Coomassie brilliant blue.

1.7. Detection of MDA content

Muscle samples were lysed with lysis buffer. The lysates were homogenized and protein concentrations were determined by BCA protein assay kit. For determination of MDA levels, we used the Jiancheng Biochemical MDA detection kit. All procedures strictly followed the kit protocol. The principle of the test is to react MDA with thiobarbituric acid and measure the adduct spectrophotometrically at 532 nm. Relative MDA contents were expressed as $OD_{532\ nm}$ adjusted by protein concentration.

1.8. Statistics

All values are expressed as means ± S.E.M. Significances of differences among groups were evaluated using one-way analysis of variance followed by last significant difference post hoc comparisons or unpaired t test. A P value of less than .05 was considered statistically significant.

2. Results

2.1. Impaired glucose tolerance and fasting plasma glucose in GK rats

Hyperglycemia is one of the defining features of type 2 diabetes. In the present study, nonobese diabetic GK rats with spontaneous hyperglycemia and glucose intolerance were used. To study hypoglycemic effect of EGCG, GK rats were tested with EGCG at 100 mg/kg/d

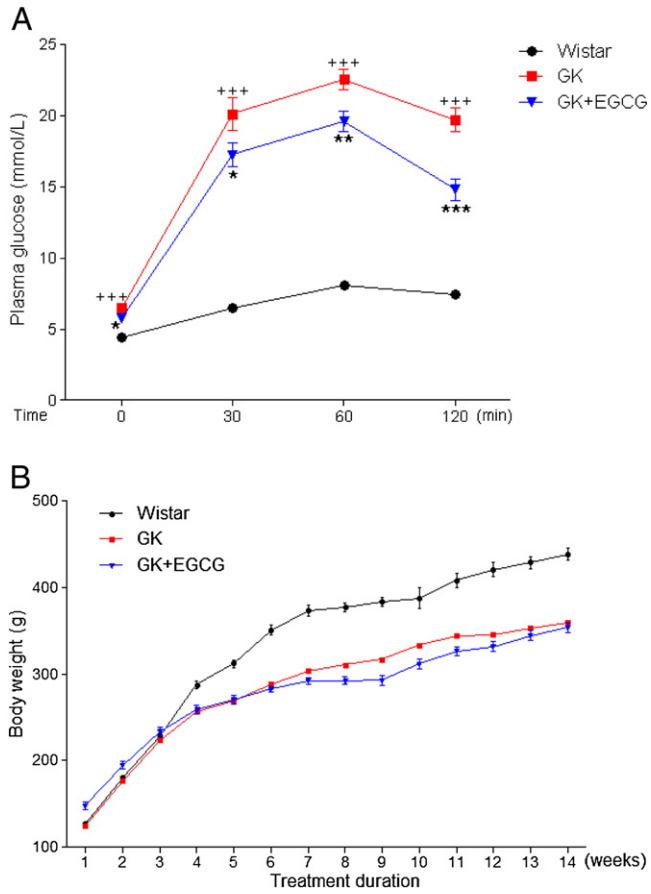

Fig. 1. Glucose intolerance and body weight. (A) Oral glucose tolerance test was conducted at the end of 13-week EGCG administration following fasting overnight. Blood was taken from the retrobulbar vein at 0, 30, 60 and 120 min after the oral glucose administration (5 g/kg body weight). (B) After 13-week EGCG administration, all rats were weighed and compared. Values are means ± S.E.M. of 12 animals in each group. +++P < .001 vs. Wistar control, *P < .05 vs. GK control, **P < .01 vs. GK control, ***P < .001 vs. GK control.

for 3 months. As illustrated in Fig. 1A, GK rats displayed greater hyperglycemic responses than Wistar rats at the indicated time points in the OGTT. Compared to those nontreated individuals, GK diabetic rats treated with EGCG for 13 weeks showed significantly lower glucose levels after glucose administration (17.24 ± 0.81 vs. 20.10 ± 1.17 mmol/L30 min, 19.58 ± 0.69 vs. 22.52 ± 0.70 mmol/L60 min, 14.80 ± 0.75 vs. 19.69 ± 0.84 mmol/L120 min, P < .001, respectively). We also compared fasting plasma glucose concentrations between the experimental groups ("0" point in OGTT curve, Fig. 1A); the fasting glucose concentration in GK diabetic rats was significantly higher than that in Wistar controls (6.44 ± 0.35 vs. 4.40 ± 0.05 mmol/L, P < .001), and EGCG treatment significantly reduced fasting glucose concentrations by 10.09% (P < .05, Fig. 1A). Furthermore, EGCG treatment induced no gain in body weight (Fig. 1B). These results indicated that EGCG could improve glucose tolerance and hyperglycemia in GK rats.

2.2. T2DM associated oxidative stress in skeletal muscle

Because diabetes is closely associated with oxidative stress [32], we used the protein carbonyl assay as a sensitive measure for protein oxidative damage, an indication of oxidative stress status. As illustrated in Fig. 2A and B, the levels of protein carbonyls in soleus muscle of GK rats were markedly higher than Wistar rats. Contents of MDA, an indicator of lipid peroxidation, showed a trend similar to protein carbonylation: MDA levels were significantly elevated in GK rats (Fig. 2C). Along with increased oxidative damage, expression of phase 2 enzymes HO1 and mitochondrially located antioxidant MnSOD were significantly augmented, whereas such alteration was not significant in NQO1 (Fig. 2D, E). Treatment with EGCG significantly reduced expression of HO1 and the content of MDA but not MnSOD (Fig. 2C, D, E); it also suppressed carbonylation (Fig. 2A, B). Skeletal muscle in GK rats presents IR as shown by the maximal insulin-stimulated Akt activation that has been widely used as an indicator for IR. Yet, the basal level of Akt is seldom studied in GK rats. Here, we observed that the basal PI3K pathway (without insulin stimulation) was activated in GK rats. Phosphorylation of Akt and its substrate GSK3β was significantly increased in GK rats, and the increase was significantly reversed by EGCG (Fig. 2F, G), consistent with previous results indicating that oxidative stress stimulates basal Akt and GSK3β phosphorylation [33]. These data suggest that significant IR is coupled with oxidative stress in soleus muscle from GK rats, and EGCG seems to reduce the effect of oxidative stress.

2.3. Oxidative-stress-associated mitochondrial loss in skeletal muscle

The mitochondrion is the major source of reactive oxygen species (ROS) during hyperglycemic conditions [34], rendering it the primary target for oxidative insult and in consequence leading to mitochondrial dysfunction [10]. In this study, we investigated the expression of the 30-kDa complex I subunit (a protein constituent of the mitochondrial respiratory chain) and VDAC1 (a mitochondrial outer-membrane binding protein) to estimate the mitochondrial content in soleus muscle. Similar to our previous results [19], GK rats had significantly lower expression of complex I and VDAC1 compared to Wistar controls, which was normalized by EGCG treatment (Fig. 3A, B). However, expression of PGC-1 and mtTFA, key transcriptional factors involved in mitochondrial biogenesis, showed no significant changes either between Wistar and GK or GK and EGCG-treated GK rats (Fig. 4A, B, C). In addition, the mitochondrial fusion-related proteins, Mfn1, Mfn2 and OPA1, showed no evident alteration between GK and Wistar rats, although interestingly the expression of Mfn2 was significantly increased by EGCG treatment and OPA1 also showed a mild increase in EGCG-treated GK rats compared with those in GK rats without EGCG treatment (Fig. 4A, B, C). These findings imply that mitochondrial loss in GK rats may not be attributed to deregulation of mitochondrial biogenesis and that EGCG's protective effects may be achieved by other mechanisms.

2.4. Up-regulated fission-involved autophagic machinery

Since mitochondria are under strict surveillance rendering them subject to autophagy at any time [24,25], it is postulated that autophagy may contribute to the loss of mitochondria in GK rats. Expression of two critical markers in the process of autophagy, LC3B and Beclin1, was determined by Western blotting (Fig. 5A, B). LC3 localizes to autophagosomal membranes after posttranslational modifications [35]. Here, both the cytosolic form and phosphatidylethanolamine (PE)-conjugated form of LC3B (LC3B-I and LC3B-II, respectively) significantly increased in GK rats vs. Wistar rats, similar to the pattern of Beclin1. Meanwhile, the ratio of LC3-II/LC3I was marginally increased in GK rats. Mitochondrial fission is indispensable for mitochondrial autophagy [25]. We next examined expression of mitochondrial dynamics-related proteins in skeletal muscle. As shown in Fig. 5, expression of DRP1 was significantly elevated in GK rats compared to Wistar controls. On the other hand, EGCG administration significantly reduced the expressional levels of Beclin1 and DRP1 (Fig. 5A, B). These results imply that mitochondria may undergo excessive autophagy in diabetic GK rats, which may be responsible for the loss of mitochondria in soleus muscle.

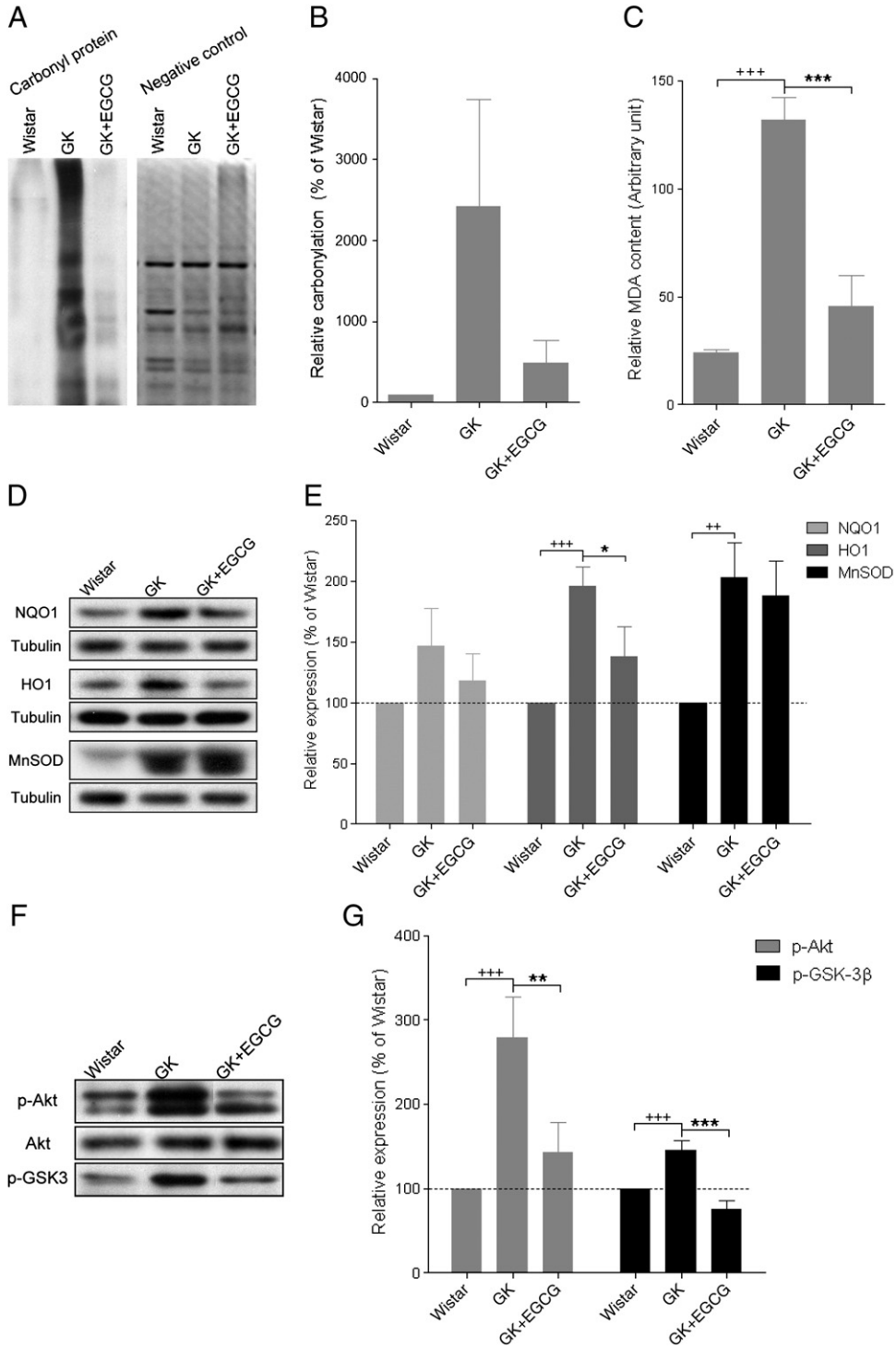

Fig. 2. Oxidative stress and basal activation of Akt in skeletal muscle. Oxidative stress was assayed by (A, B) protein carbonylation (n=6) and (C) MDA content (n=6). Expression of (D, E) NQO1 (n=6), HO1 (n=6), MnSOD (n=6) and (F, G) p-Akt (Ser 473, n=11), Akt (n=6), p-GSK3β (Ser 9, n=11) was determined by Western blotting. Expression of each protein was adjusted to α-tubulin as loading control. Values are means ± S.E.M. $++P<.01$ vs. Wistar control, $+++P<.001$ vs. Wistar control, $*P<.05$ vs. GK control, $**P<.01$ vs. GK control, $***P<.001$ vs. GK control.

2.5. EGCG reversed the activation of mitogen-activated protein kinase and p53

Mitogen-activated protein kinases (MAPKs) respond to various types of cellular stress, including oxidative stress. It is reported that ERK and JNK may act upstream of p53 in cell death subsequent to autophagy [36,37]. In this study, we examined whether the ERK/JNK-p53 pathway is involved in autophagy in GK rats. We found that phosphorylation levels of ERK1/2 and JNK were much higher in GK rats than in Wistar controls (Fig. 6A, B), but those of p38 were not increased (data not shown). These alterations are accompanied by high levels of p53 in GK rats, in contrast to Wistar controls (Fig. 6A, B). (−)-Epigallocatechin-3-gallate was able to reverse the phosphorylation of JNK but not that of ERK (Fig. 6 A, B) and, therefore, reduced autophagy.

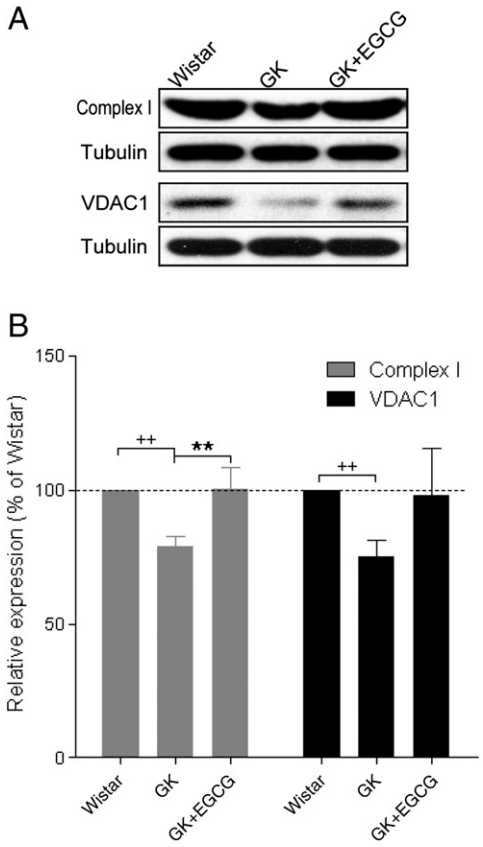

Fig. 3. Mitochondrial loss in skeletal muscle. (A, B). Expression of complex I (n=11), and VDAC1 (n=6) was determined by Western blotting. Expression of each protein was adjusted to α-tubulin as loading control. Values are means ± S.E.M. ++P < .01 vs. Wistar control, **P < .01 vs. GK control.

3. Discussion

Hyperglycemia puts diabetic subjects at greatest risk of developing diabetic complications [2]. Therefore, controlling hyperglycemia prevents the progression of complications and ameliorates patients' afflictions. Our biochemical results confirmed that GK rats have higher plasma glucose concentrations than Wistar controls in the fasting state and during OGTT. Hyperglycemic damage as a component of T2DM pathology is mediated by ROS largely produced by mitochondria [2,34]. We further examined whether oxidative stress arose due to hyperglycemia. Lipid and protein are the major targets for ROS attack. Lipid peroxidation caused by excessive exposure to ROS produces aldehydes that in turn attack protein and result in protein carbonylation. In this study, we investigated the concentration of MDA and protein carbonyls, two indicators reflecting oxidative stress. As expected, we found that total MDA and protein carbonyls were significantly elevated in skeletal muscle from GK rats. Increased lipid peroxidation and protein carbonyls present increased oxidative stress that is induced by ROS production and may subsequently lead to mitochondrial loss. Meanwhile, mitochondria-specific MnSOD and phase 2 enzymes (NQO1, HO1) were increased correspondingly. It is suggested that GK rats might develop higher levels of antioxidant defenses as an adaptive mechanism, especially in mitochondria, in an attempt to overcome mitochondrial superoxide derived from metabolic overload [10], yet still fail to counteract the overwhelming amounts of ROS present. Furthermore, in the present study, we observed a marked increase of DRP1 in GK rats, which may contribute to the elevated ROS production induced by hyperglycemia. Elevated ROS production as a consequence of hyperglycemia is known to be mediated by mitochondrial fission and mitochondrial fragmentation [38].

In this study, we also found that the contents of VDAC1 and a complex I subunit were significantly decreased in GK rat skeletal muscle. Similarly, our previous data demonstrated that mtDNA contents and mitochondrial components also were clearly reduced in GK rats [19]. Noteworthy, MnSOD and VDAC are both mitochondria proteins. MnSOD is located in mitochondria matrix and is reported to be regulated by tumor suppressor p53 and therefore affect mitochondrial ROS production [39,40]; increased MnSOD indicates elevated oxidative stress, while decreased VDAC better represents elevated mitochondrial autophagy due to its role in mitophagy. Taken together, these results strongly indicate an apparent loss of mitochondria in these animals. To better understand what causes these reductions, we checked first the impairment of mitochondrial biogenesis, since decreased PGC-1 potentially can down-regulate oxidative metabolism-relevant genes in diabetes [21,22]. Yet, in this study, expression of the transcription factor PGC-1 as well as its downstream target mtTFA showed no evident reduction in GK rats compared with Wistar rats, consistent with previous results [5,19,23]. Similarly, mitochondrial fusion proteins (Mfn1, Mfn2 and OPA1) were not altered either. Therefore, we concluded that GK diabetic rats present no obvious defects in mitochondrial biogenesis; so any hypothesis that would explain the loss of mitochondria must be attributed to another mechanism.

In the past few years, autophagy has been widely studied in multiple biological processes and in disease pathogenesis [41]. Several compelling studies have unveiled a role for autophagy in β-cells, implicating this process in diabetes [42–44]. There is little evidence pointing to insulin-sensitive tissues such as skeletal muscle, liver and adipose that may also be influenced by autophagy. Recent evidence proves that mitochondrial autophagy governs mitochondrial quality control and eliminates damaged mitochondria [24,25]. We were curious about whether mitochondrial loss was the consequence of autophagy-mediated degradation. Here, for the first time to our knowledge, we provide experimental evidence that skeletal muscle in diabetic GK rats undergoes intensive autophagy as indicated by the simultaneous increase in expression of LC3B and Beclin1. LC3B is a mammalian homologue of yeast Apg8p, and its processing represents the hallmark event for autophagosome formation during autophagy [35]. Beclin1, the yeast homologue of Apg6/Vps30, is another biomarker for the progression of autophagy [45]. That glucose or lipid-induced ROS production leads to impaired mitochondria in skeletal muscle [10] suggests that, in GK rats, damaged mitochondria eliminated during autophagy may account for the net loss of mitochondria loss in skeletal muscle. Furthermore, the fact that DRP1 was elevated in GK rats supports the hypothesis that increased mitochondrial fission may facilitate mitochondrial autophagy [24,25]. Nonetheless, whether mitochondrial autophagy in this circumstance is selective or nonselective remains to be elucidated. In addition, caspase-3 cleavage was not conspicuous (data not shown), thus ruling out the possibility that mitochondrial degradation results from caspase-dependent apoptotic cell death.

Oxidative stress is closely linked to autophagy; for example, ROS induces autophagic cell death in cancer cells [46]. The pathway involved in ROS-induced autophagic cell death remained unclear until recently, when two research groups revealed that ERK and/or JNK mediates LC3B and Beclin1 elevation. TNF-α-induced autophagic cell death was attributed to ERK/JNK-mediated p53 activation with resultant LC3B and Beclin1 up-regulation [37], and disassociation of Beclin1 and Bcl-2 was regulated by JNK, also through p53 phosphorylation [36]. Given that ERK and JNK respond to various cellular stresses such as oxidative stress, it can be speculated that ROS may activate ERK and/or JNK so as to trigger autophagy in skeletal muscle, but this remains to be determined. We found that ERK and JNK were

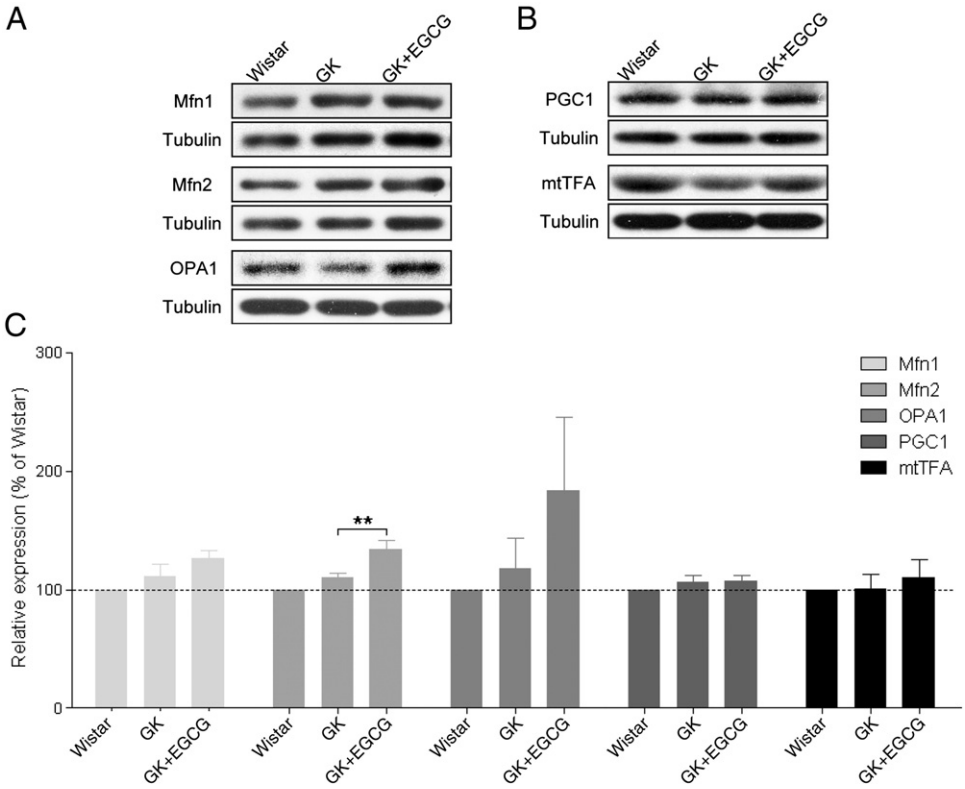

Fig. 4. Unaltered mitochondrial biogenesis in skeletal muscle. (A, B, C) Mfn1 (n=6), Mfn2 (n=6), OPA1 (n=6), PGC-1 (n=6) and mtTFA (n=6) were determined by Western blotting. Expression of each protein was adjusted to α-tubulin as loading control. Values are mean ± S.E.M. $^{**}P < .01$ vs. GK control.

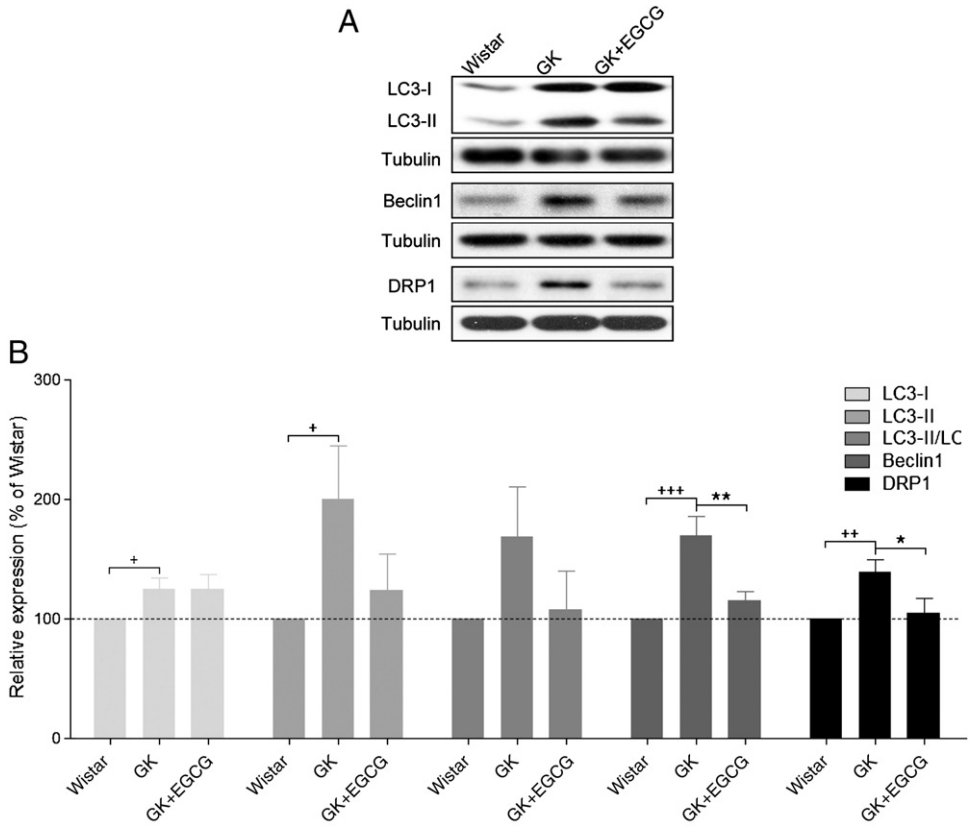

Fig. 5. Induced autophagy in skeletal muscle. (A, B) Expression of LC3B (n=10), Beclin1 (n=11) and DRP1 (n=10) was determined by Western blotting. Expression of each protein was adjusted to α-tubulin as loading control. Values are means ± S.E.M. $+P < .05$ vs. Wistar control, $++P < .01$ vs. Wistar control, $+++P < .001$ vs. Wistar control, $^{*}P < .05$ vs. GK control, $^{**}P < .01$ vs. GK control.

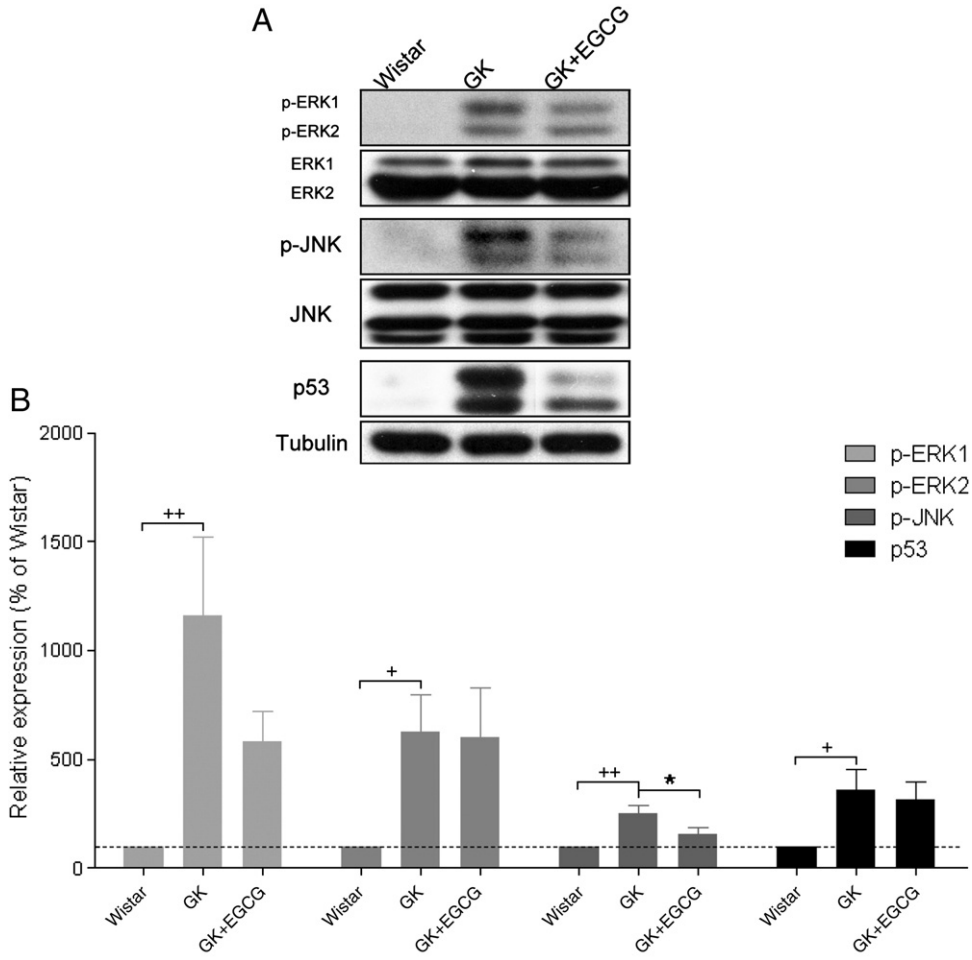

Fig. 6. (−)-Epigallocatechin-3-gallate reversed MAPK and p53 activation. (A, B) Expression of p-ERK1/2 (Thr 202/Tyr 204, n=10), ERK (n=6), p-JNK (Thr 183/Tyr 185, n=6), JNK (n=6) and p53 (n=6) was determined by Western blotting. Expression of each protein was adjusted to α-tubulin as loading control. Values are means ± S.E.M. $+P < .05$ vs. Wistar control, $++P < .01$ vs. Wistar control, $*P < .05$ vs. GK control.

activated in GK rats. Meanwhile, p53 was up-regulated, perhaps also due to elevated oxidative stress; that DRP1 was transcriptionally regulated by p53 further extends the involvement of p53 [47]. Thus, the ROS-ERK/JNK-p53 pathway may well explain the mechanism by which autophagic machinery is induced in diabetic GK rats; however, this proposal needs detailed verification.

Several studies have proven that EGCG can regulate glucose homeostasis and improve IR in hepatocytes and rodents [26–29]. In this study, EGCG is confirmed to decrease fasting glucose concentrations and improve glucose tolerance in GK rats. A possible mechanism for this hypoglycemic effect can be partly explained by the observation that EGCG repressed glucose production in an insulin-mimetic way either (a) by repressing PEPCK and G6Pase gene expression in H4IIE hepatoma cells (supported by *in vivo* evidence [28,29]) or (b) through activation of AMPK [26]. Repression of gluconeogenesis by EGCG may contribute to the maintenance of fasting glucose levels in plasma. However, the fact that basal glucose production in young and insulin-resistant elderly subjects is similar, yet their rates of insulin-stimulated peripheral glucose uptake differ [7], raises the possibility that irregular glucose infusion into peripheral insulin-responsive tissues may contribute more to glucose maintenance in GK rats. Skeletal muscle is the major site responsible for glucose disposal and may contribute most to IR [48]. Although the cause-and-effect between mitochondrial dysfunction and IR remains controversial, it is reasonable to consider that IR in skeletal muscle is connected with mitochondrial dysfunction in T2DM pathology for the following reasons: first, inherited mitochondrial dysfunction of lower mitochondrial density [5], reduced oxidative phosphorylation [6] and consequent accumulation of fatty acid metabolites [7] disturb insulin signaling through multiple cell signaling pathways [49]; second, mitochondria-mediated ROS production driven by glucose and lipid metabolic overload is able to induce IR [50,51]; and third, reduction of mitochondria weakens glucose metabolic capacity [52]. Therefore, mitochondrial dysfunction is a cardinal contributor to impaired glucose metabolism and IR.

In earlier studies, we found that mitochondrial nutrients protect mitochondria through (a) inhibiting or preventing oxidant production in mitochondria, (b) scavenging and inactivating free radicals and ROS, (c) repairing mitochondrial damage and enhancing antioxidant defenses by stimulating mitochondrial biogenesis and inducing phase 2 enzymes and (d) acting as cofactors/substrates to protect mitochondrial enzymes and/or stimulate enzyme activity. In the present study, EGCG administration reduced oxidative stress in skeletal muscle from GK rats, and this effect may be due to reduced fission of mitochondria. Astonishingly, EGCG was also able to up-regulate mitochondrial fusion machinery, especially Mfn2. It also has been reported that overexpression of Mfn2 blocks hyperglycemia-stimulated ROS [38]. These results imply that enhanced mitochondrial integrity may influence ROS production. However, whether mitochondrial integrity is directly induced by EGCG or elicited as a repair mechanism independent of EGCG stimulation [53] is unknown. As depicted in Fig. 7, we hypothesize that EGCG disrupts the vicious cycle

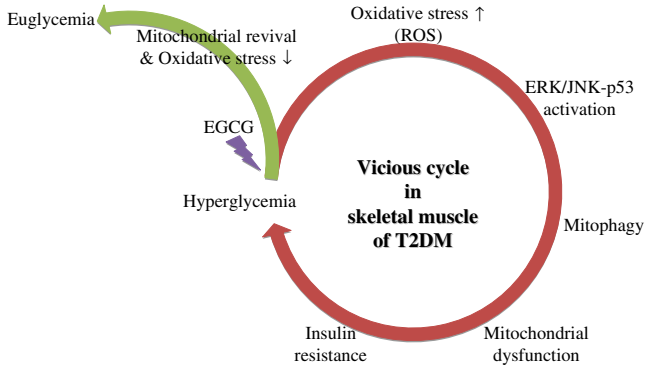

Fig. 7. A hypothetical scheme of a potential mechanism for T2DM-induced damage in skeletal muscle of GK rats. In T2DM pathology, a vicious cycle of hyperglycemia, oxidative stress and mitochondrial dysfunction is formed: hyperglycemia stimulates excessive ROS production and impairs mitochondria. The resultant oxidative stress also triggers the ERK/JNK-p53 pathway that induces mitochondrial fission and autophagy, leading to the loss of mitochondria. Mitochondrial dysfunction further worsens insulin resistance and exacerbates hyperglycemic risk. (−)-Epigallocatechin-3-gallate may, by improving mitochondrial function and reducing oxidative stress together with other mechanisms, interrupt the vicious cycle [26,27,29] and ameliorate glucose overload.

of hyperglycemia, oxidative stress and mitochondrial dysfunction by protecting mitochondria from damage and by reducing ROS production. Reduced oxidative stress may further ameliorate ROS-activated mechanisms — decreased phosphorylation of ERK and JNK, inhibition of p53 and down-regulation of autophagic machinery — and thus prevent mitochondria from degenerating. Finally, reduced oxidative stress and improved mitochondrial function together sensitize insulin signaling and enhance glucose transport for metabolism.

In conclusion, mitochondrial loss was caused by autophagy in skeletal muscle of GK rats, which was stimulated by the ROS-ERK/JNK-p53 pathway. (−)-Epigallocatechin-3-gallate effectively improved insulin resistance and glucose homeostasis in GK diabetic rats by reversing the above signaling. Thus, our findings suggest that EGCG, by regulating mitochondria-involved autophagy, might prove useful as a potential agent for treating insulin resistance.

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