Atorvastatin Prevents Ischemic Limb Loss in Type 2 Diabetes: Role of p53

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Aim: Diabetic peripheral artery disease (PAD) is prone to be aggressive and recent reports have demonstrated that p53 accumulation may be responsible for impaired wound healing in diabetes. Statins has been demonstrated to facilitate p53 degradation by activating its specific ubiquitin ligase, MDM2. The aim of this study was to determine whether atorvastatin (ATR) improves the outcome of diabetic PAD through MDM2-mediated reduction of p53.

Methods: Male KK/Ay mice (9 weeks old) were treated with ATR (2 mg/kg/day p.o.) or vehicle for 2 weeks and subjected to ischemic hindlimb operation to generate a diabetic PAD model. Incidences of amputation and changes of p53/MDM2 signaling in each ischemic limb were assessed 2 weeks after the operation (at 13 weeks of age). Effects of ATR on the insulin resistance of age-matched (13-week-old) and unoperated KK/Ay mice were assessed by the glucose tolerance test, circulating adiponectin concentration, and changes in insulin signaling (IRS-1/Akt phosphorylation).

Results: In intact KK/Ay, ATR treatment mitigated insulin resistance without affecting cholesterol levels. All diabetic PAD models exhibited autoamputation (100%); however, ATR treatment partially restored the limb loss (41.7%). The p53 expression level in the ischemic limb of ATR-treated KK/Ay was significantly decreased and MDM2 phosphorylation level was markedly increased in tandem with the activation of Akt. Hypoxia mimetic iron chelator deferroxamine promoted p53 accumulation in H9c2 myoblast cells by suppressing the Akt/MDM2 pathway, which was restored by ATR.

Conclusions: ATR was found to restore ischemic limb loss in diabetes by augmenting p53 degradation through direct activation of the Akt/MDM2 pathway in skeletal muscle.


Key words; Diabetes, Peripheral artery disease, p53, Statins

Abbreviations; Statins: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, PAD: peripheral artery disease, IR: insulin resistance, T2DM: type 2 diabetes mellitus, ATR: atorvastatin, GTT: glucose tolerance test, MDM2: murine double minute 2

Introduction

In the patient population, diabetes is one of the strongest risk factors for peripheral artery disease (PAD)\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\, and patients with diabetes are prone to a more severe outcome of the ischemic limb\(^2\)\(^,\)\(^3\)\, suggesting that normalization of p53 may contribute to amelioration of the wound repair of diabetic PAD.

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been recognized to exert...
various pleiotropic effects both in clinical and experimental settings and one of the molecular mechanisms underlying statin-mediated pleiotropic effects is activation of the protein kinase Akt pathway. It is interesting to note that statins downregulate the expression of p53 in vitro and in vivo. The expression level of p53 is regulated by the proteasomal degradation system through its specific ubiquitin ligase MDM2, the activity of which is regulated by phosphorylation by Akt. Accordingly, we hypothesized that statins may improve the outcome of diabetic PAD by reducing p53 accumulation through activation of the Akt/MDM2 degradation pathway in diabetes. In addition, considering recent reports demonstrating that statins improve insulin resistance (IR), we observed the concomitant effect of ATR on IR in the present study.

Methods

Animals

All animal experiments were carried out under the full approval of Nagoya University School of Medicine Institutional Animal Care and Use Committee. The animals were kept in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Male 9-week-old type 2 diabetic KK/Ay mice were purchased from Clea Japan Inc. Age-matched non-diabetic control mice (C57BL6) were obtained from Japan SLC Inc. Mice were fed a normal rodent diet (CMF; Oriental Yeast Co., Ltd.) and tap water ad libitum during the study period, except prior to the glucose tolerance test.

Effects of atorvastatin on insulin resistance of KK/Ay

To assess the effects of ATR on IR, KK/Ay were randomly allocated to ATR-treated (n = 8 per group) and vehicle groups (n = 8 per group). ATR (suspended in 0.5% methylcellulose solution) had been administered orally at a dose of 2 mg/kg, once daily for 4 weeks. A dose of 6.25 mg/kg atorvastatin in mice corresponds to 10 mg/day in humans for the treatment of dyslipidemia. In this study, to avoid a class effect of ATR on the cholesterol profile, mice were treated with ATR at a lower dose than for cholesterol reduction. Mice were then subjected to a glucose tolerance test and the analyzed mice were euthanized to harvest blood, visceral white adipose tissue, and limb muscle specimens.

Metabolic studies

Glucose tolerance tests (GTTs) were performed on KK/Ay mice after a 4-week period of ATR or vehicle treatment. After overnight fasting (12-16 h), glucose solutions were intraperitoneally injected (1 g/kg body weight). Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after the glucose injection using a Glutest Sensor (Sanwa Kagaku). Following the GTT, tissues (skeletal muscle and visceral white adipose tissue) were harvested and analyzed by immunoprecipitation to assess the tyrosine phosphorylation of IRS-1 using specific antibodies (IRS-1; Upstate Biotechnology; phospho-Tyr (4E10); Millipore), or immunoblotting was used to detect Akt phosphorylation using a specific antibody (phosphorylated Akt (Ser473); Cell Signaling Technology).

ELISA

Plasma levels of insulin (mouse insulin ELISA; Merckodia AB) and adiponectin (Quantikine ELISA series; R&D Systems) of intact KK/Ay in a nonfasting condition were determined using commercially available kits according to the manufacturers’ protocols.

Diabetic PAD model and effects of atorvastatin on ischemic wound repair

To test the effect of ATR on ischemic wound repair in diabetic PAD, we generated an ischemic hind limb model using KK/Ay. Mice had received ATR or vehicle for 2 weeks prior to the hindlimb operation. At 11 weeks of age, mice underwent unilateral hindlimb ischemia surgery under anesthesia with sodium pentobarbital (50 mg/kg i.p.), as previously described. In brief, an incision was made in the skin overlaying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery and the distal portion of the saphenous artery, the artery and side branches were dissected free and fully excised. Over the course of the subsequent 2 weeks, the operated mice had been treated with ATR or vehicle. At 13 weeks of age, the incidence of amputation was assessed in each operated mouse just before sacrifice, and blood and tissues were harvested at the time of sacrifice and snap-frozen.

Western blotting

Small pieces of tissue samples (skeletal muscle and visceral white adipose tissue) were snap-frozen using liquid nitrogen, and tissues were subjected to frost shattering using a Cryopress (Microteck Co., Ltd) without heat denaturation. Proteins were extracted from each powdered tissue using a cell lysis buffer (RIPA buffer) containing protease and phosphatase inhibitor cocktails (Complete mini; Roche). For cultured cells, whole-cell lysates were prepared by scraping cells into the RIPA buffer. Protein concentration in each extract
was determined using the BCA protein assay kit (Pierce). Equal amounts (20 μg) of protein were electrophoresed in a 10% polyacrylamide SDS gel followed by transfer to a polyvinylidene difluoride membrane (Immobilon, Millipore), and immunoblotting was performed. Protein bands were detected using specific antibodies as follows: phosphorylated Akt (Ser473), and phosphorylated MDM-2 (Ser186) (Cell Signaling Technology); total Akt, p53, and total MDM-2 (Santa Cruz Biotechnology); and β-actin (Abcam). Membranes were then exposed to horseradish peroxidase-conjugated anti-rabbit-IgG or anti-mouse-IgG and visualized using ECL Plus (GE). The density of each protein band was analyzed using image analysis software (Image J).

Cell culture
The myoblast cell line H9c2, derived from embryonic rat heart, has been used as an in vitro model for both skeletal and cardiac muscle\(^2\). It has been reported that there is an established in vitro model of hypoxia mimetic iron chelator deferoxamine (DFX)-induced p53 accumulation in H9c2 cells\(^2\). H9c2 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 5% CO2 and 37°C, and maintained at 60-80% confluency. Cells were pre-treated with 250 μM deferoxamine (DFX) (Sigma-Aldrich) for 24 hours and then 1 μM ATR or vehicle were added to the media followed by an additional 24 hours' incubation in the presence or absence of 1 μM Akt inhibitor-IV (Calbiochem) or 200 μM Mevalonate (Sigma-Aldrich). Following 48 hours' treatment, cells were harvested, lysed with RIPA buffer and subjected to Western blot analysis.

Statistical analysis
All values are expressed as the mean ± SEM. Differences were tested for significance with an independent sample t test and ANOVA as appropriate. A p value of 0.05 or less was considered significant.

Results
Effect of atorvastatin on insulin resistance - laboratory findings
We first evaluated the effect of ATR on basal parameters in the unoperated KK/Ay. ATR had no influence on body weight (Fig. 1A), whereas the plasma glucose levels (Fig. 1B) and insulin concentrations (Fig. 1C) of ATR-receiving mice significantly de-
clined. As shown in Fig. 1D, ATR treatment had no effects on plasma cholesterol levels (total cholesterol, triglyceride, and LDL).

**Effect of atorvastatin on insulin resistance - changes in GTT, IRS-1/Akt signaling and circulating adiponectin level**

To explore the influence of ATR on IR, changes in insulin signaling in the insulin-sensitive tissues (skeletal muscle; skM and visceral white adipose tissue; visWAT) of the unoperated KK/Ay mice were evaluated (Fig. 2A, 2B). Levels of tyrosine phosphorylation of IRS-1 (Fig. 2A) and serine phosphorylation of Akt1 (Fig. 2B) were markedly enhanced both in the skM (right panel) and visWAT (left panel) of the ATR-treated group. Indeed, the GTT revealed that the delayed glucose clearance of KK/Ay mice after glucose loading was ameliorated in the ATR group (Fig. 2C). Next, we measured plasma adiponectin levels of KK/Ay mice with or without ATR treatment (Fig. 3D). Plasma adiponectin concentration was significantly reduced in vehicle-treated KK/Ay (6551.3 ± 337.31 ng/mL; each n = 8) compared with the non-diabetic group (p < 0.01). ATR treatment increased plasma adiponectin of KK/Ay (6039.9 ± 516.47 ng/mL, n = 8) to a similar level to that of the non-diabetic group (p = 0.42).

**Atorvastatin reduces p53 accumulation in diabetic mice by activating Akt/MDM2 axis**

Next, we compared the basal expression level of p53 in skeletal muscle between intact KK/Ay and C57BL6 (Fig. 3A, 3B). with or without ATR treat-
ment. In accordance with previous reports\(^4\), there was no detectable level of p53 in the non-diabetic control. In contrast, vehicle-treated KK/Ay exhibited a marked increase in p53, which was significantly lowered by ATR treatment. Next we observed changes in Akt/MDM2 phosphorylation levels using the same specimen of KK/Ay (Fig. 3C, 3D). Both Akt and MDM2 phosphorylation levels were enhanced in ATR-treated KK/Ay.

**Atorvastatin rescues ischemic limb amputation by reduction of p53 through the activation of Akt/MDM2/ axis in type 2 diabetic mice**

Fig. 4A shows typical images of the outcome of ischemic hindlimbs in KK/Ay. All ischemic hindlimbs of vehicle-treated KK/Ay experienced autoamputation at postoperative day 14 (100%, \(n = 12\)). In contrast, no amputation occurred in the ischemic limb of operated non-diabetic mice (0%, \(n = 8\), Table 1). To elucidate the influences of the genetic background, we also observed the outcomes of ischemic hindlimbs in db/db mice, another type 2 diabetic mouse with a distinct genetic background. Age-matched db/db mice also exhibited incurable ischemic wound (66.7%, \(n = 12\), Table 1). ATR treatment partially but significantly improved the incidence of amputation (41.7%, \(n = 12\), Table 1).

To clarify the role of the p53 and Akt/MDM2 axis in ATR-mediated alleviation of ischemic amputation, we observed changes in the Akt/MDM2/p53 axis in the preserved ischemic limb of ATR-treated KK/Ay. As compared to the vehicle group, p53 expression level was markedly decreased in ATR-treated KK/Ay with a concomitant increase in Akt/MDM2 phosphorylation (Fig. 4B, 4C).

Ischemia promotes p53 upregulation through tissue hypoxia\(^22\). Next, to elucidate whether ATR may directly attenuate p53 accumulation in the ischemic limb muscle with concomitant Akt/MDM2 phosphorylation, we investigated the effect of ATR on the p53/MDM2/Akt axis using an established *in vitro* model of hypoxia-induced p53 accumulation\(^21\) (Fig. 4D, 4E). Treatment with a hypoxia mimetic iron chelator DFX (250 \(\mu M\)) induces p53 accumulation in
H9c2 myoblasts with a concomitant decrease in Akt/MDM2 phosphorylation levels, which were restored by subsequent treatment with ATR (1 μM). Akt inhibitor (1 μM) suppressed ATR-induced effects on the Akt/MDM2/p53 axis, whereas mevalonate (200 μM) had no effects, suggesting that the effect of ATR on the Akt/MDM2/p53 axis is independent from cholesterol synthesis pathway.
Table 1. Clinical outcomes of the ischemic hindlimb of PAD models of diabetic (KK/Ay, db/db) and non-diabetic control (C57/BL6) mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of limbs lost/incurable wounds (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57/BL6(non-diabetic)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>db/db</td>
<td>8/12 (66.7)</td>
</tr>
<tr>
<td>KK/Ay</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>ATR-treated KK/Ay</td>
<td>5/12 (41.7)</td>
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Discussion

The present study demonstrated that ATR ameliorates the clinical outcome of diabetic PAD, presumably through the Akt/MDM2/p53 pathway. We found a marked accumulation of p53 in the intact limbs of KK/Ay (Fig. 3) and this was further enhanced by ischemia (Fig. 4: 4.96-fold higher than the non-ischemic condition in densitometry, data not shown). All ischemic hindlimbs of KK/Ay experienced autoamputation, which was partially but significantly rescued by ATR treatment (Table 1, 41.7% incidence of limb loss) with concomitant reduction of p53 accumulation and Akt/MDM2 activation (Fig. 4). Pajjarvi et al. demonstrated that statins induced the phosphorylation of MDM2 and promote p53 degradation\textsuperscript{11}. It has been clearly demonstrated that Akt enhances the ubiquitination-promoting function of Mdm2 by phosphorylation of Ser186, which results in the reduction of p53 protein using \textit{in vitro} system\textsuperscript{13, 20} and it is interesting to note that statins activate Akt in the vascular endothelium\textsuperscript{8-10}. In the present study, we found that ATR treatment increased IRS-1/Akt phosphorylation in the skeletal muscle of intact KK/Ay (Fig. 2). Accordingly, we assessed whether ATR promotes the activation of Akt and subsequent MDM2 activation, leading to p53 degradation in the ischemic limb, which is expected to improve the clinical outcome of ischemic wounds.

A previous report\textsuperscript{6} and the present study (Fig. 3) demonstrated that p53 remains low in normal tissue because of ubiquitination and proteasomal degradation, and that ischemia promotes p53 upregulation through tissue hypoxia\textsuperscript{22}. Indeed, we consistently observed p53 upregulation in the ischemic skeletal muscle of KK/Ay (Fig. 4: 4.96-fold higher than the control as described above). Accordingly, to elucidate the direct effects of ATR on p53 accumulation in the ischemic skeletal muscle, we used the established \textit{in vitro} model of p53 accumulation induced by hypoxia mimetic DFX in H9c2 myoblasts\textsuperscript{20}(Fig. 4D, 4E). In the present study, we found that DFX increased p53 with a reduction of Akt/MDM2 phosphorylation. ATR treatment attenuated the p53 accumulation induced by DFX with a concomitant increase in Akt/MDM2 phosphorylation, which was abrogated by Akt inhibitor. Of note, mevalonate had no effect on the the effects of ATR. Collectively, these data suggest that 1) ATR may restore the hypoxia-induced increase in the p53 level by direct activation of Akt, which may be independent of its class effect as a statin on the cholesterol synthesis pathway. We also tested the impact of ATR on p53-overexpressing H9c2 cells using the adenoviral vector of p53 (\textit{supplemental figure 1}). Consistent with a previous report, upregulation of p53 increased MDM2 expression and activity\textsuperscript{24}. Indeed, ATR augmented Akt phosphorylation in p53-overexpressing cells; however, the changes in MDM2 phosphorylation and p53 reduction of ATR-treated cells were subtle under these conditions, suggesting that the direct effect of ATR on Akt and subsequent MDM2-mediated p53 degradation may be masked by MDM2 upregulation induced by p53 overexpression through the p53-MDM2 autoregulatory loop in H9c2 cells\textsuperscript{24}.

In this study, we also found that ATR restored IR in KK/Ay mice. There is ample evidence of a statin-mediated beneficial effect on insulin sensitivity\textsuperscript{25, 26}; however, the molecular mechanism remains unclear. Recently, Minamino \textit{et al.} demonstrated that p53 expression in adipose tissue crucially contributed to the development of IR\textsuperscript{27}. Indeed, we confirmed there was no detectable expression of p53 in the insulin-sensitive tissues (skM and visWAT) of non-diabetic mice in contrast with intact KK/Ay (Fig. 3). Moreover, we consistently found that ATR-treated KK/Ay exhibited improved IR with an increase in the IRS-1/Akt phosphorylation level of the insulin sensitive tissues, in which p53 level was reduced (Fig. 3A, 3B). Thus, these data indicate that ATR-induced Akt activation may alleviate IR by augmenting the degradation of p53 through the activation of MDM2, which presumably links to improved wound healing under diabetic conditions.

In addition of p53, we have to consider the alternative mechanisms underlying ATR-mediated amelioration of IR. Adiponectin has been known to be reduced in IR including T2DM\textsuperscript{28}. \textit{Fig. 2D} demonstrates that KK/Ay exhibited hypoadiponectinemia, which was restored by ATR, consistent with a previous report\textsuperscript{29}. Experimental studies suggest that adiponectin exerts a direct insulin-sensitizing effect; however, it has yet to be concluded whether adiponectin directly modulates insulin sensitivity because of the complex higher-order structure of adiponectin and in-
consistent reports regarding its putative receptors\textsuperscript{40).

**Limitations**

Our data demonstrated that ATR could rescue 58.3% of the total ischemic limb of KK/Ay from autamputation; however, we could not exclude the p53-independent mechanisms responsible for diabetic limb loss in this study. Further examination will be considered, such as exhaustive proteome analysis comparing the ATR-sensitive leg of KK/Ay to the insensitive counterpart.

The present study did not elucidate a link between the amelioration of IR and improved wound healing in T2DM. Clinical trials have emerged showing that intensive control of diabetes reduces macrovascular events\textsuperscript{31,32}; however, the impact of IR on the diabetic PAD outcome remains unclear. Our study suggests that mitigation of IR may contribute to restoration of the clinical outcome of diabetic PAD in the patient population.

In conclusion, the present study demonstrates the key role of p53 in diabetes in terms of macrovascular complication and proposes another role of statins as an option for the medical control of diabetes and its complications.

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Supplemental Figure 1
Effect of atorvastatin on p53 overexpression in H9c2 cells induced by adenoviral vector. Overexpression of p53 induced by adenoviral p53 vector (M.O.I. = 20) per se increased MDM2 phosphorylation and its expression level in H9c2 cells. Atorvastatin (1 μM) increased Akt phosphorylation level in the p53-overexpressing H9c2 cells, however, the changes in MDM2/p53 levels were subtle.