Adiponectin protects against doxorubicin-induced cardiomyopathy by anti-apoptotic effects through AMPK up-regulation

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Aims Adiponectin (APN) has been reported to protect against ischaemia–reperfusion injury and hypertrophy. However, few reports have investigated the cardioprotective effects of APN in doxorubicin (DOX)-induced cardiomyopathy; therefore, we studied the cardioprotective mechanisms of APN in this model.

Methods and results In an in vivo study, we quantified the cardiac pathohistology of C57BL/6 mice [wild-type (WT) mice], APN transgenic mice with high APN concentrations [APN transgenic sense (SE) mice], and those with reduced APN concentrations [APN transgenic antisense (AS) mice] after intraperitoneal injections of DOX (4 mg/kg) weekly for 6 weeks. The survival rate after 14 days was significantly increased in APN-SE mice (WT vs. APN-AS vs. APN-SE: 40 vs. 17 vs. 73%, $P_{<0.05}$). We assessed myocardial pathohistological changes and observed that fibrosis and apoptosis were significantly decreased in APN-SE mice compared with those of the other groups. We also assessed DOX-induced apoptotic mechanisms in vitro using cultured cardiomyocytes isolated from neonatal WT mice. The expression of adenosine monophosphate-activated protein kinase (AMPK) and anti-apoptotic factor Bcl-2 increased, but that of pro-apoptotic factor Bax decreased in cardiomyocytes treated with highly concentrated APN. The protective effects of APN were reversed by the addition of an AMPK inhibitor (dorsomorphin) to the culture medium.

Conclusion These data suggest that APN improved cardiac function through anti-apoptotic effects by up-regulation of AMPK in DOX-induced cardiomyopathy in mice.

Keywords Apoptosis • Cardiomyopathy • Heart failure

1. Introduction

Adiponectin (APN) is an adipocyte-derived bioactive factor that is abundantly present in the human plasma.1 It exists in human and mouse plasma in the form of the following oligomers: trimer, hexamer, and high-molecular-weight oligomer.2 APN works as a major anti-diabetic and anti-atherogenic adipokine. APN receptors 1 and 2 (AdipoR1 and AdipoR2) have been proved to mediate anti-diabetic effects.3 Circulating APN levels are diminished in obese individuals1 and type 2 diabetes patients.4 Previous studies have been unable to ascertain whether high plasma APN levels have cardioprotective properties in heart failure (HF) patients. Some studies have found that high APN levels are associated with increased mortality in HF patients,5,6 perhaps because high APN levels are indicative of systemic wasting in these populations.1 In this regard, low body mass index (BMI), which leads to elevated APN levels, is associated with increased mortality following the onset of HF, whereas elevated BMI, which leads to reduced APN levels, increases the risk of HF.7 In contrast, high APN levels have been associated with a lower risk of myocardial infarction (MI),8 whereas a rapid decline in APN levels following MI and persistently low APN levels could be predictive of future adverse cardiac events following MI.9 Furthermore, low APN levels have been associated with progression of left ventricular (LV) hypertrophy in hypertensive patients.10 In experimental studies, APN-
knockout (APN-KO) mice exhibited diet-induced insulin resistance,11 impaired angiogenic response to ischaemia through the suppression of excessive reactive oxygen species production,12 and excessive cardiac remodelling after pressure overload via the suppression of extracellular signal-regulated kinase activities.13 Furthermore, APN increased adenosine monophosphate-activated protein kinase (AMPK) activities in cardiomyocytes, thus preventing cardiac dysfunction.14

Although doxorubicin (DOX) is a very effective anti-neoplastic agent with a broad antitumour spectrum, its clinical use is limited by progressive and dose-related cardiotoxicity that may not manifest itself until many years after treatment.15 Two distinct phases of DOX-induced cardiotoxicity have been described: acute cardiotoxicity and chronic cardiomyopathy.16 The former typically occurs within several weeks of DOX administration and is characterized by acute inflammation. The latter appears as many as 15 years after DOX administration and is characterized by progressive LV dysfunction leading to irreversible congestive HF.17 DOX-induced HF has been attributed to apoptosis mediated by the intrinsic signalling cascade, resulting in mitochondrial dysfunction and myofibrillar degeneration.18 In animal models, studies have shown that DOX causes apoptotic cell death in cardiomyocytes.19 We also reported that exogenous nitric oxide produced an anti-apoptotic effect by suppressing caspase activity via S-nitrosylation in DOX-induced cardiomyopathy.20 Furthermore, it has been shown that the suppression of AMPK and its phosphorylation results in increased apoptosis of cardiomyocytes in DOX-induced cardiomyopathy.21

Although investigation of the mechanism of anti-apoptosis in cardiomyocytes could lead to new treatments for HF, there are currently no reports on whether APN has cardioprotective effects in DOX-induced chronic cardiomyopathy. Here, we investigated the roles of APN and APN-associated signals in DOX-induced chronic cardiomyopathy in APN transgenic mice. We also assessed the mechanisms of the cardioprotective effects of APN in this model.

2. Methods

2.1 Generation of transgenic mice

We used the same transgenic mice as described previously.22 Sense and antisense APN expression vectors were constructed by insertion of an inverted fragment of mouse APN complementary DNA into the unique EcoRI site. A Basic Local Alignment Search Tool (BLAST) analysis (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA) of the GenBank nucleotide database indicated that these sequences showed no significant homology to any other mouse genes.

2.2 Animals and experimental protocol

APN transgenic sense (APN-SE) mice (n = 30), APN transgenic antisense (APN-AS) mice (n = 30), and C57BL/6 mice as wild-type (WT) controls (n = 30) received an intraperitoneal injection of either DOX (Wako Pure Chemical, Osaka, Japan) or saline at 8 weeks of age. DOX was dissolved in sterile saline immediately before use and injected at a volume of 30 μL and a concentration of 4 mg/kg once a week for 6 weeks. The control animals were injected with an equivalent volume of saline. The animals were observed daily and weighed weekly. After 14 days from the last injection, the cardiac functions of the animals were examined by the usual methods and the animals were then sacrificed. This previously described model reproducibly introduced end-stage HF.23 All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol was approved by the Animal Research Committee of Tokyo Medical and Dental University.

2.3 Physiological studies

Blood pressure (BP) and heart rate were measured using a tail-cuff system on unaesthetized mice that had been pre-warmed for 10 min at 37°C in a thermostatically controlled heating cabinet (BP-98A; Softron, Tokyo, Japan). Transthoracic echocardiography was carried out with an ultrasound machine (Nemio; Toshiba, Tokyo, Japan) and a 13 MHz annular array transducer under light chloral hydrate anaesthesia (10 mg/kg) (Wako Pure Chemical Ind.). Hearts were imaged in two-dimensional mode in short-axis views at the level of the papillary muscles. Interventricular septal thickness (IVST), posterior wall thickness, LV end-diastolic dimension (LVDd), LV end-systolic dimension (LVDs), and the fractional shortening ratios (%FS = [(LVDd – LVDs)/LVDd] × 100) were calculated from the M-mode recordings. Each dimension was presented as the average of measurements recorded over three consecutive cardiac cycles. Measurements were made offline by two independent investigators.

2.4 Histological analysis

Hearts were removed immediately after sacrifice and fixed in 10% phosphate-buffered formalin. The fixed tissue was then embedded in paraﬃn and cut transversely into sections. These sections were stained with haematoxylin–eosin (HE) and Masson's trichrome staining (Muto Pure Chemicals, Tokyo, Japan). The area of the myocardium and surrounding tissue affected by the myocarditis (consisting of inﬂammatory cells and myocardial necrosis) was determined by computer-assisted analysis (Scion Image beta 4.0.2; Scion Corp., Frederick, MD, USA). For the analysis of LV fibrosis, six random photomicrographs were taken of the viable myocardium for each animal. The fibrosis extent and myocyte diameter in these photomicrographs was quantied by a blinded observer using the ImageJ program from NIH Image Software (National Institutes of Health, Bethesda, MD, USA). All data were analysed in a blind fashion by two independent investigators and then averaged.

2.5 Cell culture and treatment

Neonatal cardiomyocytes from 1- or 2-day-old WT mice were isolated, subjected to Percoll gradient centrifugation, and cultured in vitro as described previously.20 The cardiomyocytes were incubated in Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% calf serum (JRH Biosciences, Lenexa, KS, USA) for 24 h at 37°C.24 The cardiomyocytes were divided into five groups: the saline group (n = 6), in which only 4 μL of saline was added to the cultured medium and 1 μmol/L DOX was added to induce myocardial apoptosis after 12 h; the medium-concentrated APN (APN-medium) group (n = 6), in which 30 μg/mL APN (BioVendor Research and Diagnostic Products, Candler, NC, USA) was added and 1 μmol/L DOX was added; the high-concentrated APN (APN-high) group (n = 6), in which 100 μg/mL APN and 1 μmol/L DOX were added; the AMPK inhibitor (APN-high + AMPK-i) group (n = 6), in which a 90 min pre-treatment with an AMPK inhibitor, Compound C (dorsomorphin),25 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-phyrrazolo[1,5-a]-pyrimidine (Calbiochem, La Jolla, CA, USA), inhibited AMPK phosphorylation, after which time 100 μg/mL APN and 1 μmol/L DOX were added and the AMPK activator [saline + 5-aminomidazole-4-carboxamidine ribonucleo-side (AICAR)] group (n = 6), in which a 90 min pre-treatment with an AMPK activator, AICAR26 (Cell Signaling Technology, Danvers, MA,
USA), activated the phosphorylation of AMPK, after which time 4 μL of saline and 1 μmol/L DOX were added. Each group of control cardiomyocytes were injected with an equivalent volume of saline, without DOX. After 24 h from the last addition of saline or DOX, the cardiomyocytes were harvested from the cultured medium. For the investigation of mitochondrial-dependent death pathways, mitochondria were isolated from the cardiomyocytes using a commercially available kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The Kaplan–Meier analysis was used to examine survival differences between the groups. Differences were considered significant at \( P < 0.05 \).

### 3. Results

#### 3.1 Physiological studies and biomarkers in vivo

In all groups, serum total APN levels were significantly lower after 14 days than at the baseline. Those in the APN-SE group were about double those in the WT group. Also, those in the APN-AS group were about half of those in the WT group (Figure 1A). At necropsy, we found the following: of the WT group, 10 mice had ascites, four had lobulated enlarged livers indicative of liver congestion, and three had foamy lungs indicative of pulmonary oedema; of the APN-SE group, three had ascites, two had liver congestion, and two

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All primers used in this study are shown.
had pulmonary oedema; of the APN-AS group, 14 had ascites, six had liver congestion, and four had pulmonary oedema. The survival rate after DOX treatment was significantly higher in the APN-SE group than in the WT group and was significantly lower in the APN-AS group than in the WT group (Figure 1B).

In haemodynamic and morphological parameters, heart rates did not significantly differ among the three groups. Systolic BP significantly decreased in DOX-injected mice compared with vehicle-injected mice in all groups. Systolic BP was significantly preserved in the APN-SE group compared with the WT and APN-AS groups. The ratio of LV weight to body weight significantly decreased after DOX injection in the WT and APN-AS groups but was preserved in the APN-SE group. In contrast, the ratio of lung weight to body weight significantly increased after DOX injection in the WT and APN-AS groups but was preserved in the APN-SE group (Figure 1C). In echocardiographic studies, LVFS and IVST significantly decreased after DOX injection in the WT and APN-AS groups but were preserved in the APN-SE group (Figure 1D). The thickness of the posterior wall showed a similar tendency (data not shown).

3.2 Apoptotic myocardial changes in vivo

In HE staining of the myocardium, cardiomyocyte size significantly decreased after DOX injection in the WT and APN-AS groups but was preserved in the APN-SE group. On the other hand, the area of fibrosis seen on Masson’s trichrome staining significantly increased in the WT and APN-AS groups compared with the APN-SE group (Figure 2A).

Apoptotic cells in the in vivo cardiomyocytes detected as TUNEL-positive nuclei significantly increased after DOX injection in the WT and APN-AS groups but did not in the APN-SE group (Figure 2B).

3.3 Expression of AdipoR1, AdipoR2, and AMPK in vivo

AdipoR1 and AdipoR2 transcripts were significantly down-regulated after DOX injection. However, although AdipoR2 down-regulation was similar between the three groups, AdipoR1 was less down-regulated in the APN-SE group. The messenger RNA (mRNA) expression of AMPK-α1 and -α2 significantly decreased after
Figure 2 (A) Histological changes after 14 days in the WT (n = 6), APN-SE (n = 6), and APN-AS (n = 6) groups. (a–e) Masson’s trichrome staining. Original magnification in (a–c) is ×20. Original magnification in (d–f) is ×400. (B) Myocardium apoptosis in vivo after 14 days in the WT (n = 6), APN-SE (n = 6), and APN-AS (n = 6) groups. Bars, 50 μm. Open bars indicate the vehicle-injected mice of each group; closed bars indicate the DOX-injected mice of each group. *P < 0.05 compared with the vehicle-injected mice of each group. #P < 0.05 compared with the WT group after DOX injection.
DOX injection in the WT and APN-AS groups but increased in the APN-SE group. The mRNA expression of Bax significantly increased after DOX injection in the WT and APN-AS groups but was suppressed in the APN-SE group. In contrast, mRNA expression of Bcl-2 significantly decreased after DOX injection in the WT and APN-AS groups but not in the APN-SE group (Figure 3A).

In western blotting analyses, the protein levels of AdipoR1 and AdipoR2 decreased significantly after DOX injection. However, although the reduction of AdipoR2 was similar among the three groups, the reduction of AdipoR1 was less in the APN-SE group. The ratio of phosphorylated-AMPK-α (p-AMPK-α) to AMPK-α protein significantly decreased after DOX injection in the WT and APN-AS groups but increased in the APN-SE group. The ratio of cleaved caspase-3 to caspase-3 protein increased after DOX injection in all groups, but that in the APN-AS group was higher than that in the WT group, and that in the APN-SE group was lower than that in the WT group. The expression of Bax protein significantly increased after DOX injection in the WT and APN-AS groups but was suppressed in the APN-SE group. In contrast, the expression of Bcl-2 protein significantly decreased after DOX injection in the WT and APN-AS groups but was preserved in the APN-SE group (Figure 3B).

3.4 Apoptotic changes of cardiomyocytes in vitro

The percentage of viable cells was significantly higher in the APN-high group than in the APN-medium and saline groups. In contrast, the degree of caspase-3 activity was significantly lower in the APN-high group than in the APN-medium and saline groups. After the addition of AMPK inhibitor (AMPK-I), these changes in the APN-high group (Figure 4A). The numbers of apoptotic cells, which were detected as TUNEL-positive nuclei, significantly increased after the addition of DOX to the APN-medium and saline groups but did not increase in the APN-high group. After the addition of AMPK-I, the numbers of apoptotic cells also increased in the APN-high group (Figure 4B). In reverse transcription (RT)–PCR analyses, mRNA expression of Bax significantly increased after the addition of DOX to the APN-medium and saline groups but was suppressed in the APN-high group. In the APN-high + AMPK-I group, this expression was up-regulated as doses of AMPK-I increased. In contrast, mRNA expression of Bcl-2 significantly decreased after the addition of DOX to the APN-medium and saline groups but did not decrease in the APN-high group. In the APN-high + AMPK-I group, this expression was down-regulated as doses of AMPK-I increased (Figure 5A). In western blotting analyses, expression of Bax significantly increased after the addition of DOX to the APN-medium and saline groups but was suppressed in the APN-high group. In the APN-high + AMPK-I group, this expression was up-regulated as doses of AMPK-I increased. In contrast, expression of Bcl-2 significantly decreased after the addition of DOX to the APN-medium and saline groups but not in the APN-high group. In the APN-high + AMPK-I group, this expression was down-regulated as doses of AMPK-I increased (Figure 5B). These changes were attenuated in the saline + AICAR group vs. the saline group. Moreover, the levels of AdipoR1 mRNA expression and protein significantly decreased after the addition of DOX in the APN-medium and saline groups but not in the APN-high group. In contrast, AdipoR2 expression decreased similarly in the APN-medium, saline, and APN-high groups (Supplementary material online, Figure S1). Their expressions did not change with the use of either AMPK-I or AICAR.

3.5 Mitochondria-triggered apoptosis of cardiomyocytes in vitro

In western blotting analyses, the ratio of p-AMPK-α to AMPK-α significantly decreased after the addition of DOX to the APN-medium and saline groups vs. their controls, but these changes were not significant compared with those of the APN-high group control. This ratio in the APN-high group significantly decreased after the addition of DOX as doses of AMPK-I increased. In contrast, the ratio of cleaved caspase-3 to caspase-3 significantly increased after the addition of DOX to the APN-medium and saline groups vs. their controls, but these changes were not significant compared with the APN-high group control. This ratio in the APN-high group significantly increased after the addition of DOX as doses of AMPK-I increased (Figure 6A). Furthermore, mitochondrial cytochrome c significantly decreased and cytoplasmic cytochrome c significantly increased after the addition of DOX to the APN-medium and saline groups vs. their controls, but these changes were not significant compared with those of the APN-high group control. In the APN-high + AMPK-I group, mitochondrial cytochrome c significantly decreased and cytoplasmic cytochrome c significantly increased after the addition of DOX as doses of AMPK-I increased (Figure 6B). These changes were attenuated in the saline + AICAR group vs. the saline group.

4. Discussion

This study is the first to show that APN is cardioprotective in the context of DOX-induced chronic cardiomyopathy. We found that AMPK-dependent anti-apoptotic function is associated with cardioprotection. This conclusion was supported by the findings that the AMPK inhibitor dorsomorphin increased the pro-apoptotic factor Bax, suppressed the anti-apoptotic factor Bcl-2, and increased DOX-induced cardiomyocyte apoptosis through increased caspase-3 activity.

APN-AS mice showed deterioration of LV function following DOX-induced cardiomyopathy compared with WT mice. Conversely, APN-SE mice showed the maintenance of high plasma APN levels after DOX injection vs. attenuated detrimental LV dysfunction at 14 days in WT mice. Thus, we conclude that APN protects against the development of LV dysfunction, primarily by inhibiting the pathological apoptotic process that occurs in cardiomyocytes.

In our study, AMPK-α phosphorylated at the Thr172 residue, the activated form of AMPK, was increased in APN-SE mice compared with WT mice. The increase in the ratio of cellular adenosine monophosphate to adenosine triphosphate is a major regulator of AMPK activity.28 Recently, however, adipocyte-derived hormones also have been reported to activate AMPK.29 Most of the beneficial effects of APN appear to be mediated by AMPK-associated signalling. Our study demonstrated that the cardioprotective effects of APN after DOX-induced cardiomyopathy involve the AMPK signalling pathway. In our in vitro experiment results showed that cytochrome c was transferred from the mitochondria into the cytoplasm and then activated cleaved caspase-3 in DOX-induced cardiomyopathy. Apoptosis is known to be a tightly regulated and evolutionarily conserved
Figure 3 (A) mRNA expression levels of myocardium after 14 days in the WT (n = 6), APN-SE (n = 6), and APN-AS (n = 6) groups. (B) Western blotting analysis of myocardium after 14 days in the WT (n = 6), APN-SE (n = 6), and APN-AS (n = 6) groups. Open bars indicate the vehicle-injected mice of each group; closed bars indicate the DOX-injected mice of each group. *P < 0.05 compared with the vehicle-injected mice of each group. #P < 0.05 compared with the WT group after DOX injection.
Figure 4 (A) Analysis of cellular viability and caspase-3 activity in the APN-medium (n = 6), saline (n = 6), saline + AICAR (0.5 mM) (n = 6), APN-high (n = 6), and APN-high + AMPK-I (1 µM) (n = 6) groups. (B) Analysis of apoptosis by the TUNEL methods of DOX-treated cells in the APN-medium (n = 6), saline (n = 6), APN-high (n = 6), and APN-high + AMPK-I (1 µM) (n = 6) groups. All pictures were taken 24 h after the last addition of saline or DOX. Arrows indicate TUNEL-positive nuclei. *p < 0.05 compared with the APN-medium group. #p < 0.05 compared with the saline group. ^p < 0.05 compared with the APN-high group.
process. Mitochondria have been confirmed to play a critical role in apoptosis. Physiological and pathogenic death signals by mitochondria-dependent death pathways may result in cytochrome c release. Cytochrome c released from the mitochondria to the cytoplasm activates caspase-9, which activates the caspase-3 effector and leads to apoptotic cell death.

Our in vivo and in vitro experiment results showed that Bcl-2 was up-regulated and Bax was down-regulated after DOX injection in proportion to the doses of APN and AMPK-α phosphorylation. Members of the Bcl-2 family are key regulators of apoptosis and are divided into the following groups: (i) anti-apoptotic members including Bcl-2 and (ii) pro-apoptotic members including Bax. Anti-apoptotic Bcl-2 can interfere with cytochrome c release and suppress apoptosis progression. In response to apoptotic stimuli, Bax overexpression with conformational changes counters the death repressor activity of Bcl-2, stimulates the release of cytochrome c and other apoptogenic proteins into the cytoplasm, and ultimately initiates apoptosis.

We also observed the effect of APN in an acute DOX-induced cardiotoxicity model. The survival rate in the APN-SE group was significantly higher than the WT and APN-AS groups (data not shown).
Figure 6 (A and B) Western blotting analysis of the cytoplasm and total mitochondrial extracts of cardiomyocytes in vitro after 24 h in the APN-medium (n = 6), saline (n = 6), saline + AICAR (0.1 mM) (n = 6), saline + AICAR (0.5 mM) (n = 6), and saline + AICAR (5 mM) (n = 6), APN-high (n = 6), APN-high + AMPK-I (0.1 mM) (n = 6), APN-high + AMPK-I (1 mM) (n = 6), and APN-high + AMPK-I (10 mM) (n = 6) groups. Open bars indicate the vehicle-injected cells of each group; closed bars indicate the DOX-treated cells of each group. *P < 0.05 compared with the control of each group.
shown). Further studies are required for elucidating the mechanism of this effect.

The conformations of APN were not compared in our study. It has been reported that the proteolytically cleaved product of APN, its globular form, is present in circulation at an extremely low concentration and exhibits much more extensive biological activity than the full-length form of APN. Furthermore, AdipoR1 and AdipoR2 resulted in different expressions in our study. AdipoR1 exhibits a strong affinity for the globular form of APN and a weak affinity for full-length APN, whereas AdipoR2 exhibits an intermediate affinity for both. AdipoR1 is ubiquitously expressed in mice, whereas AdipoR2 is abundantly expressed only in the liver of mice. In our study, these differences could be associated with their phenotype. It has been shown that their ratio changes in the situation of MI, but it is still controversial whether AdipoR1 or AdipoR2 is more protective against MI. Further studies are necessary to resolve these issues by assessing AdipoR1-KO and AdipoR2-KO mice.

In conclusion, APN induces AMPK expression, activates its phosphorylation, and partially inhibits DOX-induced apoptosis in cardiomyocytes. In addition, this study demonstrates that the inhibition of AMPK phosphorylation completely reduces the cardioprotective effects of APN. Taken together, we suggest that APN is cardioprotective in DOX-induced chronic cardiomyopathy via its anti-apoptotic effects through AMPK signalling. APN could be a therapeutic target of DOX-induced cardiomyopathy and HF in mice; however, further studies must be conducted in other animals to confirm how to utilize APN in clinical HF.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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