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High-dose zoledronic acid narrows the periodontal space in rats

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Y. Okamoto, M. Hirota, Y. Monden, S. Murata, C. Koyama, K. Mitsudo, T. Iwai, Y. Ishikawa, I. Tohnai: High-dose zoledronic acid narrows the periodontal space in rats. Int. J. Oral Maxillofac. Surg. 2013; 42: 627–631.

Abstract. The aim of this experiment was to evaluate the histological effects of zoledronic acid on the periodontal space in rats. 40 male Wistar rats were divided into three zoledronic acid groups and a control group. Zoledronic acid was injected subcutaneously at doses of 10, 50, or 500 μg/kg once a week for 3 weeks. The rats were killed 1 or 9 weeks after the last injection. Histological examination of the periodontal space around the incisor tooth revealed that zoledronic acid did not inhibit tooth development. In the rats killed 1 week after treatment discontinuation, the periodontal space gradually narrowed in response to increasing zoledronic acid doses, and the changes were statistically significant according to ANOVA but not according to ANOVA with *post hoc* tests. The changes persisted in the high-dose zoledronic acid group despite zoledronic acid discontinuation, with significant differences identified by ANOVA and ANOVA with *post hoc* tests. Therefore, although zoledronic acid had an insignificant effect on tooth development, it had a significant effect on the periodontal space when high doses were administered. The results of this experiment may provide useful information for future investigations on the role of zoledronic acid in the osteonecrosis of the jaw.

Key words: bisphosphonate; zoledronic acid; osteonecrosis of the jaw; periodontal space; rat.

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Intravenous bisphosphonates are widely used as the first choice of treatment for bony metastasis of cancer and hypercalcaemia of malignancies.[1,2] Bisphosphonate treatment is effective in decreasing bony pain and serum calcaemia symptoms. Conversely, osteonecrosis of the jaw (ONJ) related to bisphosphonate treatment has been reported since 2003.[2–4] ONJ is defined as exposed necrotic bone in the maxillofacial region that persists for more than 8 weeks in a patient with current or previous bisphosphonate treatment and no history of radiation therapy against cancer in the jaws.[5,6] The highest incidence of ONJ has been associated with zoledronic acid (ZA).[7,8] Marx et al. suggested that bisphosphonates are directly responsible for ONJ because of their antiangiogenic effects.[1,4,6,9,10] The main event precipitating ONJ is dental extraction.[5,7,11] Histopathological examination revealed that bisphosphonates remarkably delayed wound healing after tooth extraction by inhibiting new bone formation.[2,5,12,13] Basi et al.[5] observed aberrant wound healing of the tooth extraction socket with decreased mineralization in a rat administered ZA and suggested that the pathogenesis of ONJ is related to high matrix metalloproteinase-9 expression and osteoclast dysfunction.

Although there are numerous clinical reports on ONJ, little information is available regarding the pathogenesis of ONJ and bony changes in the jaw after bisphosphonate treatment. Hoefert et al. reported that microcracks were present within the bones in approximately 54% of ONJ patients.[14] Takahashi et al. reported that the alveolar bone around the root of a tooth showed higher density on radiographs in ONJ patients than in age-matched

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controls.[15] Therefore, the present authors hypothesized that there are histological changes in the periodontal space, including the teeth and alveolar bone.

The aim of this study was to observe changes in the periodontal space of ZA-administered rats.

Materials and methods

40 male Wistar rats (Nihon SLC, Shizuoka, Japan: body weight 300–350 g; 10–12 weeks old) were used in the experiment. All rats were housed in cages with free access to food and water, and a 12 h light/dark cycle was maintained. All experiments were approved and performed in accordance with the guidelines for Animal Experiments Ethic Committee of Yokohama City University.

The 40 rats were randomly divided into four groups. Groups A, B, and C received ZA at doses of 10, 50, and 500 μg/kg, respectively. The rats in the control group received injections of saline instead of ZA. The time schedule of drug administration was designed according to the literature[2,13,16] with slight modifications to ensure long-term release after ZA discontinuation. Regarding the administration dosage, the dose of ZA for adult cancer patients weighing 50–80 kg was referenced.[9,17] As these patients receive 50–80 μg/kg ZA in one administration, 50 μg/kg was selected as the middle dose for use in this experiment. 10 μg/kg (a dose fivefold smaller than the middle dose) was selected as the low dose and 500 μg/kg as the high dose.

All rats received subcutaneous injections weekly for 3 weeks. All four groups were each randomly divided into short-term and long-term groups according to the length of the observation period. The rats in the short-term groups were killed 1 week after the last injection, and the rats in the long-term groups were killed 9 weeks after the last injection (Fig. 1). To distinguish these groups, the short-term groups were designated As, Bs, Cs, and Ctls for the A, B, C, and control groups, respectively, and the corresponding long-term groups were designated Al, Bl, Cl, and Ctll, respectively.

To evaluate the effect of ZA alone, no dental procedure or pharmacological therapy was performed.

Histological analysis

After the rats were killed, their mandibles were resected. Excess soft tissues were trimmed, and the remaining mandibular bones were fixed in 4% formalin.

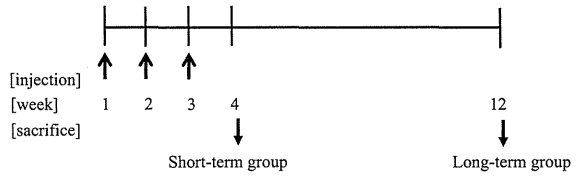

Fig. 1. Experimental design. Rats received injections of ZA or saline every Monday (↑: injection). Rats in the short-term groups were killed on the Monday of week 4, and those in the long-term groups were killed on the Monday of week 12.

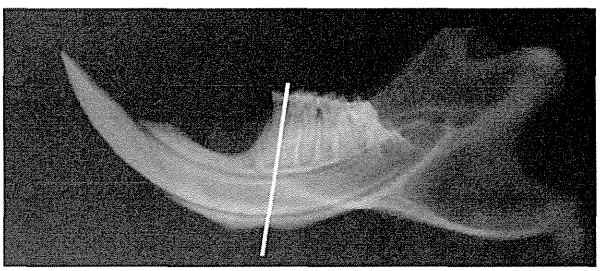

Fig. 2. To measure the periodontal space of the mandibular incisor at the same position for all specimens, cross-sections of the central portion of the first molar were used.

Following fixation, the samples were embedded in methyl methacrylate resin for histological evaluation. The embedded samples were sectioned with a microtome (30 μm thick) and stained with toluidine blue.

To measure the periodontal space of the mandibular incisor at the same position for all specimens, cross-sections of the central portion of the first molar were used (Fig. 2). On these sections, the areas of the incisor socket and the incisor were measured using MacromaX GOKO measurement software (GOKO camera Kawasaki, Japan) to calculate their cross-sectional areas (Fig. 3). To observe changes in the periodontal space, the ratio of the area of the incisor socket to that of the incisor (RSI) on the cross-section was calculated (Fig. 4).

Statistical analysis

For mean value comparisons of the incisor area and RSI between groups, ANOVA followed by Bonferroni's *post hoc* analysis for multiple comparisons was used. $P < 0.05$ indicated statistical significance.

Results

The experiment was performed without any complications, and no infection was

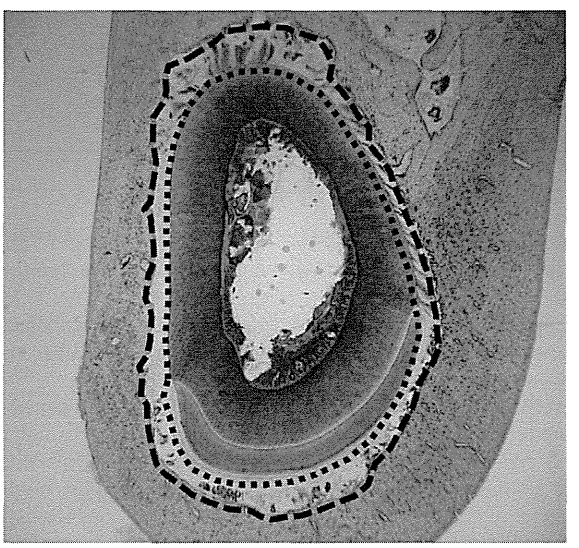

Fig. 3. The external length of the periodontal space (dotted line) and the circumference of the incisor (broken line) were measured for the statistical analysis.

$$\text{RSI} = \frac{\text{Cross-sectional area of the incisor socket [mm}^2\text{] (calculated from the external length of the periodontal space)}}{\text{Cross-sectional area of the incisor [mm}^2\text{] (calculated from the circumference of the incisor)}}$$

Fig. 4. To investigate the change in the periodontal space, the ratio of the cross-sectional area of the incisor socket to that of the incisor (RSI) was calculated.

observed in any of the rats. Histological examination revealed that neither spontaneous soft tissue necrosis nor spontaneous ONJ was observed in any of the rats.

The cross-sectional area of the incisor tended to narrow in a ZA dose-dependent manner. The narrowest and widest mean values and the standard deviations in the short-term groups were 2.340 ± 0.067 (group Cs) and 2.384 ± 0.117 mm^2 (group Ctls), respectively, whereas the corresponding values in the long-term groups were 2.389 ± 0.041 (group Cl) and 2.597 ± 0.187 mm^2 (group Ctll), respectively (Table 1). ANOVA revealed no significant differences between groups.

The mean RSI value in groups As, Bs, Cs, and Ctls was 1.480 ± 0.029, 1.444 ± 0.058, 1.429 ± 0.048, and 1.520 ± 0.053, respectively. Conversely, the values in groups Al, Bl, Cl, and Ctll were 1.456 ± 0.015, 1.481 ± 0.026, 1.413 ± 0.016, and 1.463 ± 0.006, respectively (Table 1).

In the short-term groups, the periodontal space gradually narrowed in a ZA dose-dependent manner, with statistical significance indicated by ANOVA ($F = 3.52$, $P < 0.05$) but not by ANOVA with *post hoc* tests.

In the long-term groups, ANOVA revealed a significant difference between groups with regard to the RSI, the width of the periodontal space ($F = 14.01$, $P < 0.01$). In addition, ANOVA with *post hoc* tests revealed that the RSI of the Cl group was significantly lower than that in the other groups ($P < 0.05$; Fig. 5).

Table 1. The ratio of the cross-sectional area of the socket (incisor socket) to that of the incisor (RSI). Data are presented as mean ± standard deviation. There were no significant differences among the RSI values according to ANOVA. A significant difference in RSI values was identified among the short-term groups as per ANOVA ($F = 3.52$, $P < 0.05$) but not ANOVA with *post hoc* tests. A significant difference in RSI values was identified among the long-term groups as per ANOVA ($F = 14.01$, $P < 0.01$) as well as ANOVA with *post hoc* tests ($P < 0.05$).

	Socket (mm^2)	Incisor (mm^2)	RSI (socket/incisor)
The short-term groups			
Ctls (control)	3.627 ± 0.275	2.384 ± 0.117	1.520 ± 0.053
As (ZA 10 μm/kg)	3.515 ± 0.243	2.375 ± 0.163	1.480 ± 0.029
Bs (ZA 50 μm/kg)	3.416 ± 0.346	2.363 ± 0.185	1.444 ± 0.058
Cs (ZA 500 μm/kg)	3.345 ± 0.188	2.340 ± 0.067	1.429 ± 0.048
The long-term groups			
Ctll (control)	3.799 ± 0.269	2.597 ± 0.187	1.463 ± 0.006
Al (ZA 10 μm/kg)	3.737 ± 0.136	2.567 ± 0.119	1.456 ± 0.015
Bl (ZA 50 μm/kg)	3.733 ± 0.091	2.521 ± 0.056	1.481 ± 0.026
Cl (ZA 500 μm/kg)	3.375 ± 0.067	2.389 ± 0.041	1.413 ± 0.016

Each group, $n = 5$.

Discussion

Local inflammation and connective soft tissue reactions to infection are observed in most cases of ONJ.[14,18] Stephen et al. reported that the administration of ZA with dexamethasone prior to dental extractions in rats resulted in the development of histopathological changes that were similar to ONJ in humans.[13] These findings indicate that ONJ occurs in the presence of any factor associated with bacterial infection of an intraoral wound. In the present study, the authors examined the effects of ZA, but a dental procedure that could create an intraoral wound was not performed. Therefore, spontaneous ONJ could not be observed.

In this study, narrowing of the periodontal space was observed in the high-dose ZA group; however, tooth development was not affected by systemic ZA administration. Studies reported that a topical coating of alendronate on the root surface decreased the incidence of root resorption and ankylosis in cases of tooth replantation.[19–21] In the present study, there was no root resorption and no evidence of ankylosis between the alveolar bone and

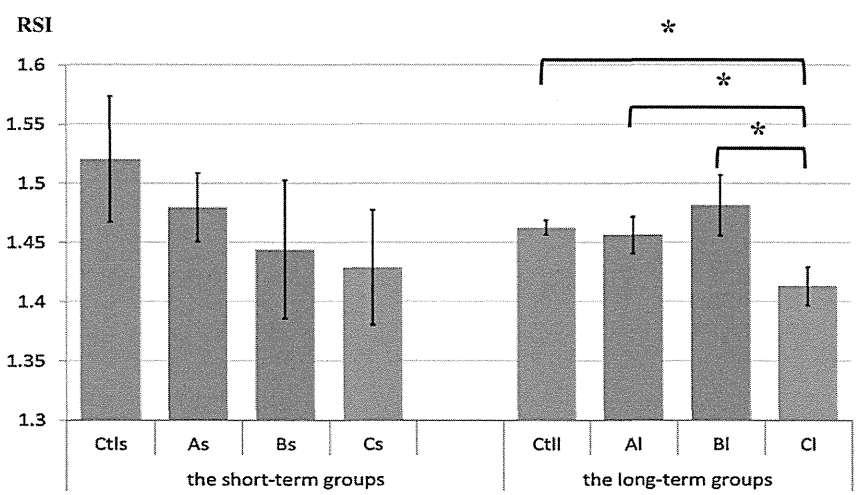

Fig. 5. The figure shows the data from Table 1 graphically. RSI is expressed as described in Fig. 4. *Significant difference as per ANOVA with *post hoc* tests ($P < 0.05$).

root, suggesting that systemic administration of ZA has an insignificant effect on tooth morphology.

In the present study, RSI decreased in response to increasing ZA doses in the short-term groups. ANOVA revealed a statistically significant difference associated with the ZA dose in the short-term group; however, ANOVA with *post hoc* tests did not confirm this significance. These results suggested that although ZA affected the width of the periodontal space, there were no significant differences between groups in this regard. The small number of samples used in the present study may explain the lack of significant differences among the short-term groups. Conversely, in the long-term groups, a significant difference was confirmed between the high-dose group Cl (500 μg/kg) and the other groups by ANOVA with *post hoc* tests, even though ZA treatment was discontinued 9 weeks previously. According to the results of both groups, the influence of ZA on the width of the periodontal space began during the early period of administration and persisted after treatment discontinuation, consequently explaining the significant difference between the high-dose group and the other groups at 9 weeks after treatment discontinuation. Then, RSI in the Bl group was higher than that in the Bs group; this finding did not hold true for the other pairs of groups. As the mean RSI values of the Bs and Bl groups had wide standard deviations, it was considered that the larger RSI of the Bl group was not a meaningful result. Considering that bisphosphonates are not metabolized and are stored within the bone for long periods of time,[2] ZA could continuously affect the width of the periodontal space in the highest dosage group. The periodontal space is tightly regulated throughout life, but certain factors such as mechanical stress and drugs may influence the width of the periodontal space. Lekic et al.[22] demonstrated that bisphosphonates decreased the width of the periodontal space by modulating the differentiation of periodontal ligament cells. Although the results in the present study were only based on histological findings, they suggested that periodontal space narrowing was induced by ZA administration, and that the narrowing was ZA dose-dependent.

The most important effect of bisphosphonates is the inhibition of bone resorption due to diminished osteoclast activity. The decrease in osteoclast activity alters the osteoclast–osteoblast interaction, according to previous reports.[2,5,8] Systematic bisphosphonate therapy would therefore be beneficial for controlling alveolar bone mass in periodontal disease.[17] Another study reported that orthodontic tooth movement is inhibited by bisphosphonates.[23]

Considering that orthodontic movement of teeth through alveolar bone requires osteoclast activity, tooth movement in the bisphosphonate-treated group was significantly less than that in the control group.[23] The findings of diminished bone resorption and remodelling at the tooth socket resulted from the altered osteoclast–osteoblast interaction. The alternation and decrease in osteoclast activity was observed to influence the differentiation of periodontal ligament cells in other studies,[20,22] suggesting that the periodontal space was narrowed by the modulated differentiation of periodontal ligament cells.[22]

Recently, ONJ associated with drugs other than bisphosphonates has been reported. One of these agents is denosumab, which is approved for use in postmenopausal women with osteoporosis and men taking androgen deprivation therapy for prostate cancer.[24] Denosumab is a humanized monoclonal antibody and antiresorptive agent that works by decreasing the activity of the nuclear factor kappa B receptor. Stopeck et al. reported that denosumab was potentially a useful medication for osteoporosis and prostate cancer; however, it induced ONJ in a similar proportion of patients as ZA.[25] Therefore, histopathological hard tissue changes caused by denosumab should be compared with those caused by ZA in further investigations to better understand the pathogenesis of ONJ.

In conclusion, ZA has an insignificant effect on tooth development and a significant effect on the width of the periodontal space, as suggested by the narrowing of the periodontal space after a short period of ZA treatment and the persistence of this effect after high-dose administration, despite ZA discontinuation. The results of this experiment may provide useful information for future investigations on the role of ZA in ONJ.

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Competing interests

None declared.

Ethical approval

Animal experiments were approved by the ethics committee at Yokohama City University (No. 10-027).

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Type 5 Adenylyl Cyclase Increases Oxidative Stress by Transcriptional Regulation of MnSOD via the SIRT1/FoxO3a Pathway

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Abstract

Background—For reasons that remain unclear, whether type 5 AC (AC5), one of two major AC isoforms in heart, is protective or deleterious in response to cardiac stress is controversial. To reconcile this controversy we examined the cardiomyopathy induced by chronic isoproterenol (ISO) in AC5 transgenic (Tg) mice and the signaling mechanisms involved.

Methods and Results—Chronic ISO increased oxidative stress and induced more severe cardiomyopathy in AC5 Tg, as left ventricular (LV) ejection fraction fell 1.9 fold more than wild type (WT), along with greater LV dilation and increased fibrosis, apoptosis and hypertrophy. Oxidative stress induced by chronic ISO, detected by 8-OhDG was 15% greater, p=0.007, in AC5 Tg hearts, while protein expression of MnSOD was reduced by 38%, indicating that the susceptibility of AC5 Tg to cardiomyopathy may be due to decreased MnSOD expression. Consistent with this, susceptibility of the AC5 Tg to cardiomyopathy was suppressed by overexpression of MnSOD, whereas protection afforded by the AC5 KO was lost in AC5 KO×MnSOD$^{+/-}$ mice. Elevation of MnSOD was eliminated by both sirtuin and MEK inhibitors, suggesting both the SIRT1/FoxO3a and MEK/ERK pathway are involved in MnSOD regulation by AC5.

Conclusion—Overexpression of AC5 exacerbates the cardiomyopathy induced by chronic catecholamine stress by altering regulation of SIRT1/FoxO3a, MEK/ERK and MnSOD, resulting in oxidative stress intolerance, thereby shedding light on new approaches for treatment of heart failure.

Keywords

Adenylyl cyclase; Adrenergic; Cardiomyopathy; Oxidative Stress

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Disclosures

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Introduction

Adenylyl cyclase (AC) is a key regulator of health and longevity in organisms ranging from yeast to mammals.[1–5] In the heart AC is a critical link in sympathetic control and beta adrenergic receptor (beta-AR) signaling and therefore plays a fundamental role in mediating not only baseline cardiac function, but also the cardiac response to stress, e.g., in the pathogenesis of heart failure. Type 5 AC (AC5) is one of two major isoforms in heart, the other being type 6 AC (AC6). For reasons that remain unclear, whether AC5 is protective or deleterious in response to cardiac stress is controversial, particularly with respect to the signaling mechanisms involved, and whether these mechanisms are shared by AC6. It is generally accepted that cardiac-specific AC5 overexpressed (AC5 Tg) mice exhibit enhanced cardiac performance,[6] which follows from the role of AC, which generates cyclic AMP upon beta-AR stimulation resulting in increased cardiac contractility and heart rate. However, the extent to which altered AC5 regulation is protective with chronic stress remains controversial. Prior studies examined whether overexpression or disruption of AC5 in the heart could affect the progression of cardiomyopathy induced by overexpression of Galphaq and beta1-AR. This was accomplished by mating overexpressed Galphaq and beta1-AR with AC5 Tg or AC5 knockout (KO) mice. These studies found that AC5 Tg rescued Galphaq cardiomyopathy,[6] but not beta1-AR cardiomyopathy,[7] and AC5 KO mice failed to rescue cardiomyopathy in Galphaq mice.[8] In addition, AC5 KO mice rescued cardiomyopathies from chronic pressure overload,[9] chronic catecholamine stress,[10] and aging.[1]

Since beta-AR signaling, of which AC is central, plays a key role in the pathogenesis of heart failure and since beta-AR blockade therapy is widely used in patients with heart failure, but that therapy is still far from perfect, it becomes critical to reconcile the controversy and understand the role of AC in the heart in the development of cardiomyopathy and heart failure, which would eventually be of clinical importance. Accordingly, this was the overall goal of the current investigation. We first examined the extent to which manganese superoxide dismutase (MnSOD) regulation and oxidative stress were altered in AC5 Tg at baseline and in response to chronic beta-AR stimulation, since it is known that beta-AR stimulation increases oxidative stress,[11, 12] and that MnSOD is upregulated in AC5 KO mice.[1] The results of the experiments with bigenic mice (AC5 Tg × MnSOD Tg and AC5 KO × $MnSOD^{+/-}$) led us to elucidate the signaling mechanisms linking AC5, MnSOD and oxidative stress, and the involvement of the SIRT1/FoxO3a pathway. The SIRT1/FoxO3a pathway was selected to investigate, because MnSOD is upregulated in the AC5 KO mouse, which lives longer than wild type (WT)[1] and FoxO3a is the transcriptional factor most closely related to the anti-oxidative protective effects associated with longevity, as shown in several models: *C.elegans*,[13, 14] rats[15] and human quiescent cells.[16] The final goal was to investigate whether this pathway is regulated specifically by AC5, or whether it is common to all AC signaling in the heart, which would mean that these mechanisms were shared by the other major cardiac AC isoform, AC6.

Methods

Mouse Models

Generation of AC5 Tg mice was described previously.[17] AC5 KO × $MnSOD^{+/-}$ mice were generated by crossing AC5 KO mice with MnSOD heterozygous mice. AC5 Tg × MnSOD Tg were generated by crossing AC5 Tg mice with MnSOD Tg mice (From Jackson Laboratory, Stock ID: 009438). To produce catecholamine cardiomyopathy, ISO was delivered to 3–5 month old Tg mice, bigenic mice and corresponding control littermates for 7 days at a dose of 60 mg/kg/day with a miniosmotic pump (ALZET model 2001, DURECT Corp, Cupertino, California) as described.[10] The severity of the cardiomyopathy was

assessed by echocardiographic measurements of LV ejection fraction and LV end diastolic and end systolic diameter and histopatholical measurements of myocardial fibrosis, apoptosis and myocyte cross sectional area. For the Tempol treatment group, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (Tempol, Sigma) was administered to AC5 Tg mice by dissolving it in drinking water at a concentration of 1mmol/L for 1 month prior to chronic ISO infusion to block oxidative stress. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition 2011). This study was approved by the Animal Care and Use Committee at New Jersey Medical School.

Experimental procedures

All techniques are described in more detail in Supplemental materials with references to previous work with these techniques. Experimental procedures included: adenoviral construction (Figure S2), physiological studies,[10] primary culture of neonatal rat ventricular myocytes,[18] AC assay,[10] immunoprecipitation, western blotting,[1] quantitative RT-PCR,[18] 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA assay, chemiluminescent assay for superoxide production,[19] subcellular fractionation, luciferase activity, Chromatin Immunoprecipitation (ChIP) assay[15] and histological analyses (apoptosis, fibrosis and cell size).[20]

Statistical analysis

Normally distributed data were presented as mean±SEM. Otherwise, data were summarized using the Median and range. When the data were normally distributed, we used Student's unpaired t-test to compare two independent groups; otherwise, the difference was tested using the Mann-Whitney U test. For a comparison of three or more groups, one-way ANOVA was used if the sample population was normally distributed and within-group variances were approximately equal. The Student-Newman-Keuls test was used for post-hoc analysis. For data that did not meet the ANOVA assumptions, the Kruskal-Wallis test was applied and post hoc testing was carried out using the Mann-Whitney U test with Bonferroni correction. The Bonferroni correction factor is 3 for Figures 1, 5F and 5G. GraphPad-Prism 5.0 (GraphPad-Software, San Diego,CA), SPSS 20.0 (SPSS Inc, Chicago, IL) and SAS 9.3 (SAS, Research Triangle, NC) were used to perform the statistical analyses. P-values less than 0.05 defined statistical significance.

Results

AC5 Tg Mouse Model and Cardiomyopathy Induced by Chronic Isoproterenol (ISO)

AC5 protein expression, assessed by western blot analysis, was increased 26-fold in AC5 Tg (Figure S1A). Basal AC activity was increased 13-fold in AC5 Tg mice hearts compared to WT, and was increased 10-fold with forskolin compared to WT (Figure S1B). The AC5 Tg exhibited increased left ventricular ejection fraction (LVEF), p=0.0009, without ISO (WT=73(67–74)%; AC5 Tg=78(75–81)%) and heart rate was not significantly different, p=0.3176, (WT=337(325–465) bpm; AC5 Tg= 442(355–500)bpm). The increase in LVEF in response to an ISO challenge was similar in AC5 Tg and WT mice(Figure S1C).

Chronic ISO infusion induced more severe cardiomyopathy in AC5 Tg compared with WT, i.e., LVEF was lower, p=0.0058, in AC5 Tg (45(30–49)%) compared to WT (54(47–58)%). Actually the decline in LVEF was even more significant, since that takes into account the different baseline levels where LVEF was higher in AC5 Tg and fell to a lower level, p=0.0021 (Figure 1A). In addition, the LV dilated more in AC5 Tg mice than WT (Table S1). Similarly, chronic ISO induced more fibrosis (2.0-fold) and more myocyte apoptosis (2.8-fold) in AC5 Tg mice compared with WT (Figure 1B and 1C). There was also more LV

hypertrophy, as measured by myocyte cross sectional area, but the increase (1.2-fold) was not as great as with fibrosis and apoptosis.

Overexpression of AC5 Increased Oxidative Stress

After chronic ISO stimulation, AC5 Tg mice exhibited 19% more GSSG content, an indicator of oxidative stress, than WT littermates (Figure 2A). Consistent with this, AC5 Tg mice had 15% more oxidative stress-induced DNA damage compared with WT mice after chronic ISO stimulation detected by 8-OHdG ELISA (Figure 2B). In AC5 overexpressed neonatal myocytes, superoxide production was approximately 2-fold more than in the control group (Figure 2C). AC5 knockdown (KD) myocytes increased cell survival with H_2O_2 treatment (Figure 2D). MnSOD is part of a mechanism that might be responsible for the opposite response of AC5 overexpressed (OE) and AC5 KD towards oxidative stress, since MnSOD is up-regulated in AC5 KO mice.

AC5 Down-Regulates MnSOD

By western blotting, the protein expression of MnSOD was reduced 38% in AC5 Tg mice compared with WT (Figure 3A). On the cellular level, 26% less MnSOD was detected in AC5 OE myocytes and MnSOD protein was increased a 2-fold and mRNA a 3.6-fold in AC5 KD myocytes (Figure 3B, 3C and 3D). The data demonstrated that AC5 regulated the protein and mRNA expression level of MnSOD, which altered MnSOD function.

MnSOD Overexpression Ameliorated Chronic ISO Cardiomyopathy in AC5 Tg

We increased MnSOD in AC5 Tg using a bigenic (AC5 Tg × MnSOD Tg) mouse. The cardiac specific MnSOD Tg mice had a 20-fold increase in SOD activity in the heart.[21] Baseline LVEF was similar in AC5 Tg × MnSOD Tg mice (85(84–89)%) and AC5 Tg mice (78(75–81)%). After chronic ISO, the LVEF of bigenic mice decreased significantly less (Figure 1A, Table S1), p=0.0033, to 74(66–77)%, than in AC5 Tg (45(30–49)%) mice (Figure 1A, Table S1). The increases in LVEDD and LVESD were also no longer greater than observed in WT (Table S1), and the increases in fibrosis and apoptosis, observed in AC5 Tg on chronic ISO, were no longer observed in the bigenic mice (Figure 1B and 1C). Similarly, Tempol, which also protects against oxidative stress, rescued the adverse effects of the AC5 Tg after chronic ISO stimulation, i.e., the LVEF in AC5 Tg with ISO and Tempol (63(43–69)%) was higher than with ISO in AC5 Tg without Tempol (45(30–49)%). Thus, these data demonstrated that down-regulation of MnSOD in AC5 Tg mice is a key mechanism mediating the exacerbated cardiomyopathy induced by chronic ISO.

Down-regulation of MnSOD Eliminated Protective Effects of AC5 KO under Chronic Catecholamine Stress

To investigate whether MnSOD was important for the protective effects of AC5 KO mice, we crossed the AC5 KO mice with MnSOD heterozygous KO mice. Previously, we reported that the AC5 KO mice were protected against catecholamine stress.[10] This was confirmed in the present study in a small cohort, where the fall in LVEF was less in the AC5 KO than WT with chronic ISO. This protection was lost in the bigenic mice, where LVEF after chronic ISO was decreased to 50(43–60)% (n=6), which was almost identical to the LVEF in the WT mice (53(39–62)%, n=11) (Table S1). Fibrosis, an indicator of the cardiomyopathy with chronic ISO was increased similarly in WT (2.69(1.64–3.84)%) and AC5 KO × $MnSOD^{+/-}$ mice (2.92(2.32–3.85)%) compared with AC5 KO mice with chronic ISO (1.13(0.81–1.37)%).

AC5 Regulated MnSOD Transcriptionally through the SIRT1/FoxO3a Pathway

Since down-regulation of MnSOD is the key mechanism mediating the enhanced cardiomyopathy of AC5 Tg mice, we investigated the molecular pathway responsible. The mRNA level of MnSOD in AC5 KD myocytes was 3.6-fold higher than those infected by a control adenovirus, Ad-LacZ (Figure 3D), which suggested that transcriptional factors may be involved in the regulation of MnSOD, with FoxO3a a likely target, as noted earlier. To determine whether FoxO3a directly regulated MnSOD, we first examined the localization of FoxO3a in the AC5 KD neonatal myocytes. Immunostaining and western blotting detected more FoxO3a in the nucleus of AC5 KD myocytes compared with the control group (Figure 4A and 4B). Similarly in tissue, more FoxO3a was detected in the nucleus of AC5 KO mouse heart compared with WT (Figure 4C).

Next, we tested the transcriptional activity of FoxO3a using a luciferase assay by transfecting a FoxO3a luciferase vector, with a promoter containing 3 repeats of the forkhead response element (FRE), into neonatal myocytes.[22] When AC5 was knocked down in myocytes, a 2.5-fold increase in luciferase activity was observed (Figure 4D). To examine whether FoxO3a increased the native MnSOD gene directly, we performed the ChIP assay on native MnSOD in the H9C2 rat cardiac myoblast cell line infected with Ad-LacZ and Ad-shAC5, respectively. We found 1.9-fold more FoxO3a binding to the specific FoxO-binding element within the MnSOD promoter region (Figure 4E) in AC5 KD myocytes. The data demonstrated that knock down of AC5 causes FoxO3a to localize to the nucleus and associate with the MnSOD promoter.

FoxO is known to be regulated by acetylation, a modification removed by the NAD^+-responsive, metabolic sensor SIRT1.[23] To test whether AC5 regulated SIRT1 directly; protein expression of SIRT1 was detected in AC5 KO mice hearts. SIRT1 was significantly up-regulated in AC5 KO mice hearts (Figure 5A). Consistent with the adult heart data, SIRT1 was up-regulated in AC5 KD myocytes and down-regulated in AC5 OE neonatal myocytes (Figure 5C).

To test whether this pathway was unique to AC5, we also examined AC6 KO, the other major cardiac AC isoform. In AC6 KO, SIRT1 was not up-regulated (Figure 5B). Paralleling SIRT1 expression, MnSOD was not up-regulated in the AC6 KO hearts. Thus, the up-regulation of SIRT1 and MnSOD expression was not due to a general response to AC activity.

Given the apparent link between SIRT1 and FoxO3a, we found that the level of acetyl-FoxO3a in the heart of AC5 KO and AC5 Tg mice correlated inversely with the expression of SIRT1 (Figure 5D). In AC5 KD myocytes, SIRT1 was shown by co-immunoprecipitation (IP) to bind directly to FoxO3a (Figure 5E). These data suggest that FoxO3a is regulated by deacetylation in cardiac tissue and this is controlled by AC5 activity. To further investigate the role of SIRT1, we treated AC5 KD and the control group with nicotinamide, a sirtuin inhibitor, and then tested the transcriptional activity of FoxO3a in the control group. Nicotinamide did not change the activity in the control group, but reduced the transcriptional activity of FoxO3a to almost the same level in AC5 KD group as in the control group (Figure 5F). To investigate if the elevation of luciferase activity of FoxO3a contributed to MnSOD expression, we determined MnSOD protein levels in AC5 KD myocytes. The MnSOD level in nicotinamide treated AC5 KD myocytes was down-regulated to a similar level as the control group (Figure 5G). These data indicate that the increased transcriptional activity of FoxO3a and MnSOD expression in AC5 KD myocytes is due to increased SIRT1 activity. In summary, AC5 inhibited SIRT1 activity, which consequently decreased the interaction between SIRT1 and FoxO3a, thus decreasing MnSOD expression, resulting in less tolerance towards stress (Figure 6).

MnSOD also Regulated by MEK/ERK Signaling

We previously reported that both MEK/ERK/MnSOD and Akt pathways were involved in the protective mechanism of AC5 KO.[1, 10] The elevation of MnSOD in myocytes infected with AC5 KD adenovirus was eliminated by both the MEK inhibitor (PD98059)(Figure S3B) and sirtuin inhibitor (nicotinamide) (Figure 5G), suggesting both the MEK/ERK and SIRT1/FoxO3a pathway are involved in MnSOD regulation. Furthermore, we examined the activity of Akt (represented as p-Akt/Akt) in AC5 Tg mice after chronic ISO. In contrast to AC5 KO mice, the activity of Akt was lower in AC5 Tg than in WT mice (Figure S4), indicating that the AC5 Tg failed to activate this protective mechanism induced by chronic ISO stress. Different from MnSOD basal regulation by AC5 (Figure S3A), Akt is involved in mediating the cardiac effects after chronic ISO stimulation.

To demonstrate that MnSOD is the only anti-oxidant gene targeted by the AC5 regulated Sirt1/FoxO3a pathway, we examined the expression of catalase, another downstream target of FoxO3a,[24, 25] and found no differences in the hearts among WT, AC5 KO and AC5 Tg mice (Figure S5).

Discussion

Since the conclusions from previous studies examining the extent to which cardiac overexpression or deletion of AC5 affects the development of cardiomyopathy have been controversial[6, 7, 17, 26] and no prior study examined the effects of cardiac overexpression of AC5 on the development of cardiomyopathy, the goal of the present investigation was to do just that, using the model of chronic catecholamine stress. The results indicate clearly that cardiac over-expression of AC5 increases the severity of the cardiomyopathy induced by chronic catecholamine stress, resulting in more severely compromised LV function and increased LV dilation, cardiac fibrosis and apoptosis. It is well recognized that catecholamines increase oxidative stress, which in turn, induces necrosis and results in cardiac fibrosis,[27–30] which is an important mechanism mediating the decrease in function observed, not only in the cardiomyopathy induced by chronic ISO, but also in all cardiomyopathies. Although the most common cause of necrosis is myocardial ischemia, it can also result from an imbalance between myocardial oxygen supply and demand, particularly in the subendocardium, and there is also non-ischemic necrosis,[27] all of which leads to a reduction in contractile units in the heart and increased fibrosis, which interferes with cardiac contraction. ISO also induces necrosis in myocytes in culture, independent of myocardial blood supply.[27, 31, 32]

There is another reason for controversial results in literature, i.e., it is not always possible to extrapolate linearly between in vivo and in vitro work and between Tg and KO models. A major strength of this investigation was the use of both Tg and KO models for AC5, which alleviated the criticisms that the high level of overexpression of AC5 in the Tg model, overwhelmed other mechanisms. In addition, finding reciprocal data in the KO and Tg model strengthens the conclusions.

Since MnSOD protects against oxidative stress in AC5 KO mice[1] and since oxidative stress has been implicated in catecholamine induced cardiomyopathy,[11, 27, 33] our hypothesis was that the adverse effects of AC5 overexpression in the heart are mediated by enhanced oxidative stress, primarily through an MnSOD mechanism. Confirming this hypothesis we found a 36% decrease of MnSOD expression in AC5 Tg mice and greater oxidative stress induced DNA damage. To further confirm our hypothesis that the reduced MnSOD was responsible for the enhanced oxidative stress, we restored MnSOD to the AC5 Tg mice by mating them with MnSOD Tg mice. The bigenic mice no longer responded to chronic ISO with more severe cardiomyopathy. To further confirm our hypothesis, we also examined

whether reducing MnSOD eliminated the protection afforded to AC5 KO mice. Accordingly, we also mated $MnSOD^{+/-}$ mice with AC5 KO mice and then subjected the bigenic mice to chronic ISO stimulation. The bigenic mice were no longer protected from chronic catecholamine stress. Thus, the level of expression of MnSOD was responsible for the difference in responses to chronic catecholamine stimulation from WT in AC5 Tg and AC5 KO mice, and the opposite responses in AC5 Tg and AC5 KO mice. It was important to use the KO model in parallel with the AC5 Tg model, to avoid complicating influences derived from increasing gene expression to a high level. More importantly, the elimination of the protective effect in AC5 KO × $MnSOD^{+/-}$ mice is direct evidence that indicates the importance of MnSOD in the AC5 regulatory pathway. Other studies have found that oxidative stress is an important mechanism mediating several different cardiomyopathies,[28–30, 33, 34] and Dai et al., showed the importance of mitochondrial oxidative stress.[35] However, the signaling pathways have not been elucidated.

In this connection, it was previously shown that impaired mitochondrial function in cardiac myocytes from $Sod2^{+/-}$ mouse hearts is associated with a reduction in MnSOD activity[36], which might suggest that the same phenomenon might occur in the AC5 Tg, where MnSOD activity is reduced, and that this may reduce cardiac function. However, we observed increased LV function at baseline in AC5 Tg and impaired function with chronic ISO. This apparent conundrum can be explained by the complex interaction of mechanisms controlling cardiac function in normal animals in vivo and more so in Tg animals. Since AC5 is a direct downstream target of the beta-AR, the increased basal LV function is a result of amplifying signals from the beta-AR, and not due to reduced MnSOD expression. Chronic ISO stress in AC5 Tg leads to the accumulation of oxidative stress, and an imbalance between oxygen supply and demand in the heart, leading to necrosis, apoptosis and fibrosis, which consequently reduced LV function and resulted in cardiomyopathy.

We then investigated the signaling pathway by which AC5 regulated MnSOD expression. In AC5 KD myocytes, we detected a significant increase in MnSOD mRNA, which suggests that AC5 also regulated MnSOD in a transcriptional manner. As noted earlier, the experiments demonstrating the role of MnSOD in mediating the enhanced cardiomyopathy with chronic ISO stress in AC5 Tg led us to investigate the SIRT1/FoxO3a pathway, in view of its protective role against oxidative stress associated with aging in *C. elegans*,[13, 14] rats[15] and human quiescent cells.[16] In our study, we demonstrate for the first time the importance of AC5, an up-stream gene of the SIRT1/FoxO3a complex regulating MnSOD in cardiomyopathy. We also found activation of SIRT1 and FoxO3a in AC5 KD myocytes, whereas inhibition was detected in AC5 OE myocytes, suggesting that AC5 inhibits SIRT1 and FoxO3a activity, resulting in an impaired anti-oxidant system and induced cell death, suggesting that overexpression of SIRT1 and/or nicotinamide mononucleotide (NMN) or nicotinamide riboside treatment[37] should be able to counteract the adverse effects of increased AC5 in the setting of chronic catecholamine cardiomyopathy. These conclusions are based in part on the acetylation experiments, which have the limitation of using the immunoprecipitation technique.[38] A future direction will be to utilize a specific antibody for acetyl FoxO3a when it is available, which would permit a more definitive conclusion.

FoxO3a is known to regulate MnSOD transcriptionally, protecting cells from cellular oxidative stress.[16] FoxO activity is regulated, in turn, by SIRT1, which also exerts favorable effects on oxidative stress resistance in cardiac myocytes.[23] An interaction between the cyclic AMP/PKA pathway and Sir2 (an ortholog of SIRT1) in yeast has been reported.[39] Recently a few studies indicated cyclic AMP/PKA dependent pathways of SIRT1 activation,[40,41] which seem to be at variance with our findings. However, there are at least 4 important differences between these studies and ours. These studies were conducted in cancer, skeletal muscle, or hepatic cells, whereas we examined cardiomyocytes. Secondly,

our findings are related only to AC5; it is conceivable that other AC isoforms, even AC6, the other major isoform in the heart could induce different regulation. Indeed, this is what we observed. Since in the studies by Noriega and Gerhart-Hines, forskolin was used to stimulate AC, which will activate all AC isoforms, this may have influenced AC6, which regulates AC activity to a greater effect than AC5 in the heart. Fourthly, our study was conducted under conditions of chronic activation or inhibition of the cyclic AMP/PKA pathway, e.g., in AC5 Tg and KO, and measured the change in protein expression of SIRT1, whereas the prior studies utilized more acute activation of cyclic AMP/PKA with forskolin, which activated SIRT1, either through the induction of its transcription or through SIRT1 phosphorylation. Finally, the results of our investigation relating AC5 to the SIRT1/FoxO pathway is consistent with other studies showing that SIRT1/FoxO protects the heart against oxidative stress, and that MnSOD plays an important role.[42, 43] It has been shown that overexpression of SIRT1 no longer promotes cell survival when MnSOD was eliminated[42] and nuclear translocation of FoxO induced transcriptional up-regulation of MnSOD, which protected the heart from myocardial infarction.[43] In addition, we found translocation and activation of FoxO3a by SIRT1, consistent with a report in *C. elegans*.[23] Since FoxO3a regulates several molecules involved in the cell cycle and oxidative stress, e.g., catalase[24, 25] it is possible that MnSOD is not uniquely targeted. However, we found that catalase expression was not different in hearts from WT, AC5 Tg and AC5 KO (Figure S5), supporting the concept that MnSOD is uniquely regulated by FoxO3a with relation to AC5. Deacetylation of FoxO3a should activate the transcriptional activity as described previously.[23] As shown in Figure 5F and 5G, inhibition of SIRT1 activity reduced the FoxO3a transcriptional activity and MnSOD expression in AC5 KD myocytes. Since MnSOD is one of the important down-stream targets of FoxO3a, it is expected that the binding affinity to the MnSOD promoter increases as FoxO3a is activated.

It is important that we found the signaling mechanisms differed in the two major cardiac AC isoforms, AC5 and AC6. There have been several instances of AC isoform differences within organs. AC1, AC5, and AC8 all are major isoforms in brain, AC1 and AC8 play an important role in memory and learning,[44, 45] but not AC5. Similarly, AC1 KO and AC5 KO mice are resistant to pain stress,[46, 47] but not AC8. Also there are apparent differences in AC5 and AC6 regulation in the heart, showing that the AC5 KO is protected against stress,[9, 10] but not AC6 KO.[48]

We previously found that the MEK/ERK pathway regulated MnSOD in the AC5 KO.[1] In the current investigation we found that MnSOD was upregulated similarly in AC5 KO mice with vehicle and with ISO (Figure S3A) and that both a MEK inhibitor and a sirtuin inhibitor blocked the elevation of MnSOD in AC5 KD myocytes (Figure S3B, 5G), suggesting that both of these pathways are involved in the regulation of MnSOD by AC5.

In summary, this study examined for the first time the effects of overexpression of AC5 on the response to cardiac stress. In contrast to conflicting results from prior studies in AC5 Tg,[6, 7] the results of the current investigation indicate clearly that overexpression of AC5 is deleterious in response to cardiac stress. We also demonstrated a new pathway for cardiac dysfunction mediated by AC5; cardiac overexpression of AC5 exacerbates the cardiomyopathy induced by chronic catecholamine stress through a mechanism inhibiting SIRT1 and FoxO3a, which decreases MnSOD transcription. The impaired antioxidant system elevates the intercellular oxidative stress level with chronic ISO stimulation, inducing more cell death and resulting in augmented cardiac dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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