Chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy

Jennifer G. Duncan, Rajashree Ravi, Linda B. Stull, and Anne M. Murphy

Departments of Anesthesiology and Critical Care Medicine and Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland

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Duncan, Jennifer G., Rajashree Ravi, Linda B. Stull, and Anne M. Murphy. Chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy. Am J Physiol Heart Circ Physiol 289: H1512–H1518, 2005. First published April 29, 2005; doi:10.1152/ajpheart.00168.2005.—Heart failure is a clinical syndrome associated with elevated levels of oxygen-derived free radicals. Xanthine oxidase activity is believed to be one source of reactive oxygen species in the failing heart. Interventions designed to reduce oxidative stress are believed to have significant therapeutic potential in heart failure. This study tested the hypothesis that xanthine oxidase activity would be elevated in a mouse model of dilated cardiomyopathy and evaluated the effect of chronic oral allopurinol, an inhibitor of xanthine oxidase, on contractility and progressive ventricular dilation in these mice. Nontransgenic and transgenic mice containing a tropomyosin I truncation were treated with oral allopurinol from 2–4 mo of age. Myocardial xanthine oxidase activity was threefold higher in untreated transgenic mice compared with nontransgenic mice. Analyses of myofilament proteins for modification of carbonyl groups demonstrated myofibrillar protein damage in untreated transgenic mice. Treatment with allopurinol for 2 mo suppressed xanthine oxidase activity and myofibrillar protein oxidation. Allopurinol treatment also alleviated ventricular dilation and preserved shortening fraction in the transgenic animals. In addition, cardiac muscle twitch tension was preserved to 70% of nontransgenic levels in allopurinol-treated transgenic mice, a significant improvement over untreated transgenic mice. These findings indicate that chronic inhibition of xanthine oxidase can alter the progression of heart failure in dilated cardiomyopathy.

oxidative stress; myofilament proteins

Despite advances in pharmacology and technology, heart failure remains a significant cause of morbidity and mortality. There is mounting evidence that oxidative stress due to increased production of reactive oxygen species (ROS) may play an important role in the pathogenesis of myocardial dysfunction in heart failure [see review by Singal et al. (29)]. Multiple investigators have shown that ROS formation is increased in congestive heart failure (8, 21). Whether this increase in ROS is associative or a significant contributor to the pathogenesis of heart failure remains unclear. The source of ROS in heart failure is in part due to production of the superoxide anion radical (O2·−) by the mitochondria (15) and xanthine oxidase (XO). XO and xanthine dehydrogenase (XDH) are both isoenzymes of xanthine oxidoreductase (XOR). These enzymes catalyze the reduction of hypoxanthine and xanthine to uric acid, releasing O2·−, O2·− byproducts, such as hydrogen peroxide and peroxynitrite, can negatively impact cardiac function (3, 12). XO is the predominant source of O2·−. Recent evidence suggests that XDH functions in part as an NADH oxidase and through this activity is also capable of generating additional O2·− (14, 27). XO activity or protein content has been found to be elevated in the failing heart (5, 6, 9, 16). Furthermore, elevated levels of uric acid are associated with increased morbidity and mortality in heart failure (1, 11, 20).

Previous studies have reported an augmentation of cardiac muscle contractility with acute XO inhibition. In a model of cardiac reperfusion injury, Perez et al. (24) and Kogler et al. (16) have reported that perfusion of isolated rat hearts with allopurinol or its metabolite, oxypurinol, inhibitors of xanthine oxidase activity, augmented Ca2⁺ responsiveness of both failing and nonfailing myofilaments, thereby improving contractility. Short-term inhibition of XO with intravenous allopurinol has been demonstrated in animal and human heart failure to improve mechanical efficiency and in some reports contractile function of the myocardium (5, 9, 34). Recent data have suggested that chronic inhibition of XO improves survival and cardiac function in posts ischemic cardiomyopathy (10, 32). Interestingly, studies in rat models of ischemia-reperfusion injury suggest that myofibrillar protein oxidation may contribute to contractile dysfunction (4). The role of XO inhibition in progressive nonschematic cardiomyopathy has not been studied.

A transgenic (TG) mouse model of dilated cardiomyopathy with carboxy-terminal truncation of tropomyosin I (TnI) to TnI1−193 has previously been established (22). In addition to being a model for a myofilament protein defect, these mice are an excellent model of a progressive dilated cardiomyopathy. Previous studies of these mice have demonstrated impaired maximal Ca2⁺-activated tension of the cardiac myofilament, with associated systolic and diastolic dysfunction, and progressive left ventricular remodeling with dilation (22). Furthermore, these studies have demonstrated improved systolic function using a Ca2⁺-sensitizing agent (EMD 57033), suggesting that this model may be particularly amenable to drugs that improve myofilament Ca2⁺ responsiveness (30).

The current study tested the hypotheses that 1) this TG mouse model of dilated cardiomyopathy would have increased myocardial XO activity, and 2) oral treatment with allopurinol at an early stage would alter cardiac muscle force generation and thus improve contractility and alleviate ventricular dila-
tion. This article reports that chronic XO inhibition with allopurinol in a mouse model of dilated cardiomyopathy delays heart failure progression by improving cardiac muscle force generation and preserving cardiac function. The evidence reveals that cardiac myofilaments in TG mice with dilated cardiomyopathy have significant oxidative damage that is suppressed by allopurinol therapy.

**MATERIALS AND METHODS**

**Mice.** TG mice with cardiac expression of a truncated TnI have been created as previously described (22). Nontransgenic (NTG) control mice were littermates of the TG mice.

**Experimental protocol.** All experimental protocols were approved by the Animal Care and Use Committee of Johns Hopkins University. Mice were divided into four treatment groups. Beginning at 2 mo old, TG (n = 14) and NTG (n = 8) mice were treated with either allopurinol (26 mg/dl) (n = 11: 6 TG, 5 NTG) in the drinking water or unmodified drinking water (n = 11: 8 TG, 3 NTG). Mice were serially studied with echocardiography from 2–4 mo of age. An additional 24 mice were given either allopurinol (n = 9: 4 TG, 5 NTG) or regular water (n = 15: 7 TG, 8 NTG) and utilized for studies of muscle mechanics after 1 mo of treatment.

**Echocardiography.** All mice in the initial group had echocardiography performed (Agilent Sonos 5500, 15-MHz probe, 3-cm depth) in a blinded fashion at baseline (2 mo), 3 mo, and 4 mo of age. Mice were sedated using 3% isoflurane for 90–120 s and then kept sedated with 0.6–1% isoflurane delivered via a mouse ventilator. Rectal temperature was monitored and mice were externally warmed with a heat lamp to a constant temperature of 37.5°C. In the parasternal short-axis view, measurements were taken in M mode of left ventricular ejection fraction (LVED), left ventricular end-systolic dimension (LVES), septal and left ventricular wall thickness, and R-R interval. Heart rate during echocardiography ranged from 478 to 583 beats/min. Fractional shortening (FS) was then calculated using the equation [(LVED – LVES)/LVED × 100]. Each set of measurements was taken three times on each mouse and averaged.

**XO activity assay.** XO/XOR activities were measured in each mouse group by using the spectrofluorometric assay described by Beckman et al. (2) and Kogler et al. (16). Briefly, the assay measures the conversion of pterin to isoxanthopterin (IXPT). Frozen heart tissue (25–50 mg) was homogenized 1:4 (wt/vol) in ice-cold 10 mM HEPES (Beckman et al. (2) and Kogler et al. (16)). Briefly, the assay measures allopurinol (26 mg/dl) (n = 80°C) hearts from TG mice, NTG mice, and their allopurinol-frozen hearts, and the entire procedure was carried out at 4°C. Baseline measurements were taken by mixing 25 μl of Tris buffer containing 15–20 μl of protein, an equal volume of 12% SDS was added, followed by addition of 10 μl of dinitrophenylhydrazine (DNPH) solution. Aliquots of myofilament-enriched fraction were derivatized with DNPH for various time points. The reaction was stopped at 0, 2, and 5 min by adding neutralization solution. The contents were mixed thoroughly and electrophoresed on 4–12% Bis-Tris gels and transferred onto nitrocellulose. The membrane was blocked in 1% BSA in PBS containing 0.05% Tween (PBST) for 1 h, followed by incubation with anti-DNP antibody (1:100 dilution in PBST containing 1% BSA) for 1 h, and was then washed with wash buffer (PBST), incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit, 1:300 dilution in PBST) for 1 h, and washed several times with wash buffer. The blots were developed with a chemiluminescence detection system (Amersham Biosciences). Densitometry was performed with Image J for Windows (NIH image analysis software; http://rsweb.nih.gov/ij).

Equal protein loading in each lane was achieved by determining protein concentrations using the DC protein assay kit (Bio-Rad). For comparison between samples, the densities of bands corresponding to actin on the Oxyblot were normalized to that of myosin light chain 2 (Mycosamie blue-stained gels to optimize for any differences in protein loading. The values are expressed as percentages of untreated NTG group values.

**Muscle studies.** Muscles were prepared and studied as described previously (31). Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital. After intracardiac heparinization, hearts were rapidly excised and placed in Krebs-Henseleit buffer. In addition, 20 mM 2,3-butanedione monoxime (BDM) was added to the dissection buffer to prevent cutting injury. The aorta was cannulated and blood thoroughly washed out. Thin, uniform, nonbranched trabeculae from the right ventricle were carefully dissected. Dimensions of the muscles were measured, and cross-sectional area was calculated by assuming an ellipsoid shape. Dimensions were comparable in each group. Muscles were mounted between a force transducer and a hook connected to a micromanipulator. Muscles were perfused with the same Krebs-Henseleit buffer without BDM at 22.5°C and stimulated at 0.5 Hz. Extracellular Ca2+ concentration ([Ca2+]o) was raised, and muscles were allowed to stabilize. Developed force was measured over a variety of [Ca2+]o values from 1 to 7 mM.

**Data analysis.** Results from all experiments are reported as means ± SE. Echocardiographic changes over time within individual mouse groups were analyzed using one-way analysis of variance (ANOVA) with each mouse serving as its own control. One mouse in the untreated TG group died before the final echocardiogram; the echocardiographic data recorded at baseline and 1 mo of treatment were still included in the ANOVA. Echocardiographic comparisons between mouse groups and the XO activities and Oxyblot densities were analyzed using an unpaired t-test. Muscle mechanics data were compared using two-way repeated-measures ANOVA. Differences were considered significant at a P value <0.05.
RESULTS

**XO/XOR activity is profoundly increased in cardiomyopathy.** XO and XOR activity was assessed in heart homogenates of TG and NTG mice from both the untreated and allopurinol groups after 2 mo (Fig. 1). XO activity alone was remarkably threefold higher in the TG mice (0.002 ± 0.0002 nmol·mg⁻¹·min⁻¹) compared with NTG mice (0.0007 ± 0.0001 nmol·mg⁻¹·min⁻¹, P = 0.002). The total XOR (combined XO and XDH activities) was also markedly higher (P = 0.003) in untreated TG hearts (0.005 ± 0.0004 nmol·mg⁻¹·min⁻¹, n = 5) compared with NTG myocardium (0.001 ± 0.0002 nmol·mg⁻¹·min⁻¹, n = 5). Oral administration of allopurinol was able to significantly reduce the XO/XOR activity (XO = 0.0002 ± 0.00009 nmol·mg⁻¹·min⁻¹, XOR = 0.0004 ± 0.0001 nmol·mg⁻¹·min⁻¹, P < 0.0001) in the TG hearts (n = 4) to a level less than or comparable to that in NTG controls. These findings indicate a striking upregulation of XO activity in early dilated cardiomyopathy. Allopurinol-treated NTG mice did not have a significant change in XO activity; however, there was a trend toward decreased total XOR activity with allopurinol (P = 0.06), reflecting the inhibitory properties of the medication even in normal animals.

**Allopurinol prevents oxidative protein damage in cardiac myofilaments.** Oxidative stress is known to induce protein carbonylation on the side chains of amino acids (25). To determine whether increased XO activity and its inhibition with allopurinol specifically affected myofibrillar protein oxidation, we extracted the myofibrillar proteins and quantified protein carbonylation using the Oxyblot technique. Figure 2 shows that oxidation of myofilament proteins as quantified by actin carbonylation was increased in TG mice and was attenuated by treatment with allopurinol (n = 4 in each group). Although several proteins in the myofilament-enriched fraction were significantly oxidized in TG mice, this blot (Fig. 2A) focuses on the actin band, because it was consistently the most prominent band. The reactive carbonyl groups in TG mice were profoundly increased up to 252.8 ± 53.9% that in untreated NTG mice (P < 0.05) whether measured at derivatization time 0, 2, or 5 min with DNPH. Oral treatment of TG mice with allopurinol markedly attenuated the myofilament oxidation at all time points studied (50.5 ± 8.7, 53.4 ± 20.5, and 78.4 ± 31.4% of untreated NTG mice at 0, 2, and 5 min, respectively, P < 0.01) compared with untreated TG mice. This allopurinol-induced reduction in protein oxidation was less than that in the untreated NTG mice (normalized value of 100%, P < 0.05; cumulative data depicted in Fig. 2C).

**Chronic XO inhibition attenuates ventricular dilation and preserves cardiac function.** Echocardiograms were performed on TG and NTG mice from untreated and allopurinol-treated mice after 1 and 2 mo of treatment. There were no significant differences in heart rate or in left ventricular wall or septal thickness in any of the mouse groups (data not shown). In addition, at the time the mice were euthanized, there were no differences in heart weight-to-body weight ratios in any mouse group (data not shown). Allopurinol had the greatest longitudinal impact on LVED. Figure 3A depicts individual mouse changes in LVED over 2 mo, demonstrating that LVED increased significantly (P = 0.003) over time in untreated TG animals (n = 8 at 1 mo, n = 7 at 2 mo of treatment), whereas allopurinol-treated animals (n = 6) had no significant change in LVED over the 2 mo of treatment. Untreated TG animals had an average LVED of 0.313 ± 0.007 cm at baseline, and after 2 mo, the LVED increased significantly (P = 0.0005) to 0.363 ± 0.009 cm. In contrast, allopurinol-treated animals had an average LVED of 0.331 ± 0.01 cm at baseline, and after 2 mo, the average dimension was 0.344 ± 0.008 cm. Allopurinol had the most impact on cardiac function as shown by echocardiogram after 1 mo of treatment (Fig. 3B). LVED, LVES, and FS were significantly worse in the untreated TG animals (n =
Fig. 2. TG mice have significantly higher oxidized myofibrillar proteins, measured as protein carbonyls in Oxyblot under optimal conditions and at 3 time points for derivatization. The myofilament protein oxidation is significantly attenuated in allopurinol-treated TG counterparts. A: representative Oxyblot from untreated and allopurinol-treated (Allo) hearts is depicted at the time points studied. Although several proteins in the myofilament-enriched fraction are significantly oxidized in TnI1–193 TG mice, this blot focuses on the actin band, because it was consistently the most prominent band. B: representative example of a Coomassie-stained gel that was performed to normalize the actin bands for loading. C: cumulative data depicting increased actin carbonylation in untreated TG animals and attenuation with allopurinol therapy. Results represent percentages of untreated NTG (means ± SE) as determined by densitometry of the level of actin protein carbonyls (n = 4 in each group) in untreated NTG, TG, and allopurinol-treated TG and NTG littermates. Normalization to the untreated NTG was performed by comparing samples prepared at the same time and run on the same blot. The percent increase in the untreated TG compared with the NTG control was relatively consistent when measured at each of the 3 derivatization times.

Fig. 3. Allopurinol prevents progression of left ventricular (LV) dilation and preserves cardiac function. A: individual mouse mean left ventricular end-diastolic dimension (LVED) is depicted over the 2-mo treatment course (from 2 to 4 mo of age). Untreated TG mice (n = 8 at 1 mo, n = 7 at 2 mo of treatment) had progressive LV dilation over the 2-mo treatment time course (*P < 0.01), whereas treated TG mice (n = 6) had no change in LVED over time. B: LV dimensions and percent fractional shortening (FS) are depicted after 1 mo of allopurinol therapy, as documented by echocardiography. LVED, left ventricular end-systolic dimension (LVES), and FS in treated TG mice (striped bar, n = 6) were not significantly different from LVED, LVES, and FS in untreated NTG mice (solid bar, n = 3), whereas untreated TG mice (stippled bar, n = 8) had significantly larger LVED and LVES and smaller FS than NTG mice. All results are means ± SE determined by averaging 3 different echocardiographic measurements in each mouse.
Cardiac muscle force generation is increased in allopurinol-treated mice. To characterize the improvement in cardiac function observed with allopurinol treatment, we examined developed twitch tension in isolated cardiac muscle over a range of \([\text{Ca}^{2+}]_o\) values in TG and NTG mice from each treatment group after 1 mo of treatment. A previous study demonstrated that these TG mice have significantly reduced maximal \(\text{Ca}^{2+}\)-activated twitch tension without alterations in intracellular \(\text{Ca}^{2+}\) transients compared with NTG mice (17), indicating a decreased responsiveness to \(\text{Ca}^{2+}\). In the current study there was again a 40% reduction in developed twitch tension in untreated TG mice (19.2 ± 1.5 mN/mm²) compared with NTG mice (43.9 ± 4.3 mN/mm², \(P = 0.0004\); Fig. 4). Allopurinol treatment restored developed twitch tension to 70% of that in untreated TG mice (33.6 ± 3.4 mN/mm²), which represents a significant improvement (\(P = 0.002\)) from baseline TG cardiac muscle force. Thus oral administration of allopurinol resulted in a substantial increase in contractile function in the failing TG myocardium.

**DISCUSSION**

Chronic heart failure is a pathological condition that may be associated with oxidative stress. Therapeutic interventions that modulate oxygen-derived free radicals have tremendous potential to alter clinical disease. The present study demonstrates that early oral treatment with an XO inhibitor in an established mouse model of progressive cardiomyopathy preserves cardiac function, an effect correlated with prevention of myofibrillar protein oxidation. There are several notable findings in this study. First, XO activity was increased threefold in this mouse model of dilated cardiomyopathy. Second, oxidation of actin was dramatically increased in the failing TG mouse and could be prevented by chronic treatment with allopurinol. Finally, oral allopurinol was able to preserve cardiac function in the TG heart and augment contractile force in TG muscles.

The origin and time course of XO activity in heart failure remains unknown. There is significant variability in the reported detection of XO within the myocardium of certain mammalian species, including humans (7, 28, 35). The observed increase in XO activity in the current study is striking and certainly suggests that oxidative stress occurs in the early stages of heart failure. Previous work has evaluated XO activity in animal models or human patients with symptomatic failure (5, 9, 16, 26). In contrast TnI_{1-193} TG mice exhibit a mild-moderate phenotype of heart failure and are asymptomatic at this young age. Although the time course of XO activation was not studied, it is notable that these mice had only mildly reduced FS and modest chamber dilation at the time they were euthanized for XO assays (4 mo old). However, they had a significant increase in XO activity and inhibition of the activity delayed progression of disease. This implies that XO plays an important role in disease progression even in asymptomatic animals.

In this mouse with altered TnI, it is likely that XO activity plays an exacerbating role to the underlying functional defect. The modification of TnI in these mice mimics the proteolyzed TnI that has been seen in humans with end-stage ischemic cardiomyopathy (23). It is likely that the increase in XO activity in these TG animals is not directly related to the functional alteration in TnI but, rather, a result of another as yet unidentified mechanism for enzyme activation in heart failure. For example, XO activity may increase as a result of an uncoupling of energy and force known to occur with this myofibrillar defect (13). An increase in ATPase activity with concomitant decrease in contractile force may lead to significant alterations in energy demands. XO is the rate-limiting step in purine metabolism, and this alteration in energy demand...
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may necessitate an increase in XO activity to maintain metabolic homeostasis. Alternatively, it is possible that the increase in XO activity is at least in part due to endothelial dysfunction known to occur in other models of heart failure (19). The current study reports increased XO activity in a whole heart homogenate from mice. Whether XO is derived from the cardiocyte, endothelial cell, or both remains undetermined. Further studies to identify the subcellular localization of XO and potential alterations in enzyme localization during disease will be important in better understanding the pathophysiological role of XO in heart failure.

Allopurinol therapy has an important impact on ventricular remodeling. The echocardiographic data suggest that inhibition of XO with allopurinol in the TG animals had some impact on global function early in the treatment, because FS was improved in the treated mice after 1 mo of therapy but a sustained improvement was not observed. However, there was a more profound impact on cardiac remodeling, because ventricular dilation was prevented in the treated animals over a longer period of time. The greater impact of allopurinol on cardiac contractility at 1 moo of therapy could be related to the truncation defect in TnI in this mouse model. This truncation creates a significant functional alteration in TnI known to negatively impact cardiac function (22). It is likely that the functional defect in this model cannot be completely altered by allopurinol in the long term. However, the major purpose of this study was to evaluate whether allopurinol used orally had an impact on cardiac function and remodeling in a chronic model of nonischemic failure. The extent to which allopurinol will be effective over many months remains to be studied.

The relationship between increased XO activity and improved contractile function with allopurinol has suggested that free radical damage contributes to disease pathogenesis, but the exact mechanism by which allopurinol exerts its effect has not been demonstrated previously. The present observations demonstrate a direct effect of allopurinol therapy in preventing myofibrillar protein oxidation. Oxidative modification of actin in particular is likely to cause significant mechanical dysfunction given its critical role in the contractile machinery and as suggested by the cardiomyopathies associated with its mutation (33). The effect of XO inhibition on maximum Ca2+ -activated twitch tension both in our study and in other models of failure (16, 24) is likely related to the direct effect of allopurinol in preventing contractile protein oxidation. Interestingly, allopurinol had no significant effects on protein oxidation in NTG mice, suggesting a differential effect of allopurinol in the failing myocardium. A similar finding was noted recently by Kogler et al. (16), who found that oxyuricase used alone enhanced cardiac muscle twitch tension in both control and spontaneously hypertensive, heart failure-prone (SHHF) rats without alterations in intracellular Ca2+ amplitudes; this inotropic effect was most pronounced in failing myocardium. It is important to note that in the present study only evaluated protein carboxylation. Other types of oxidative damage not studied also may contribute to myofilament dysfunction. The use of allopurinol early in heart failure may be important in preventing potentially irreversible oxidation of myofibrillar proteins and the detrimental effects on cross-bridge cycling and contractility.

This study sought to evaluate the novel use of allopurinol chronically in early, nonischemic cardiomyopathy. Furthermore, we report a new potential mechanism for allopurinol in the prevention of oxidative protein damage to critical contractile proteins. In summary, the data demonstrate a direct link between chronic inhibition of XO activity and preservation of cardiac function. The data show that XO activity is elevated in early asymptomatic stages of cardiac dysfunction, that increased XO activity correlates with oxidative damage to critical contractile proteins, and that chronic inhibition of XO activity is possible with oral administration of allopurinol and results in improved contractile function. Collectively, these findings have significant and exciting implications for treatment of patients with chronic heart failure.

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REFERENCES


