**Chronic Treatment With AllopurinolBoosts Survival and Cardiac Contractility in Murine Postischemic Cardiomyopathy**

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**Abstract**—Oxidative stress is a hallmark of systemic illnesses, including heart failure. Nevertheless, the overall importance of radical production in the heart remains conjectural; is it merely a marker of illness, or can intervention alter the progression of disease? This question was addressed by blocking xanthine oxidase (XO), a superoxide-generating enzyme, that is upregulated in animal models of heart failure. In a randomized prospective trial design, we administered the XO inhibitor allopurinol orally to mice that had undergone massive myocardial infarction (MI). Cardiac XO activity was elevated in untreated mice after MI; allopurinol suppressed the XO activity to levels comparable to those in sham-operated mice. Eighty-one percent of untreated mice died of advanced heart failure over 2 to 4 weeks of follow-up. Survival doubled in the allopurinol-treated mice, whereas cardiac contractile function (both in vivo and in isolated muscle) was markedly improved. Response to isoproterenol was restored to near-normal levels in the allopurinol group but was attenuated in untreated mice. Oxidative modifications to proteins were prevented in the allopurinol-treated mice. Our findings indicate that targeted blockade of just one source of oxidants, XO, impacts dramatically on the progression of postischemic cardiomyopathy in mice and prevents oxidative protein modifications. *(Circ. Res. 2004;95:1005-1011.)*

**Key Words:** heart failure ■ mice ■ myocardial contraction ■ oxygen

Heart failure, a condition that affects up to 8 million Americans, is accompanied by excessive free radical production. However, it is not clear whether the relationship is associative in nature or, alternatively, whether oxidative stress figures prominently in the pathogenesis of heart failure. Xanthine oxidase (XO), a superoxide-generating enzyme, is upregulated in animals and in humans with heart failure. Acute administration of the XO inhibitor allopurinol improves the mechanical efficiency of the failing heart; contractility increases, whereas oxygen consumption paradoxically decreases. Direct XO inhibition in cardiac muscle leads to increased force generation by the myofilaments at any given level of activator calcium. Despite the striking and distinctive acute effects of allopurinol, it remains unclear whether chronic treatment with an XO inhibitor is beneficial in the treatment of heart failure. This study is different from any previous work because XO inhibition by allopurinol was continuous for 28 days after myocardial infarction (MI) and was delivered orally, which is a clinically relevant delivery method. The goal of this study was to determine if the acute effects of allopurinol could be sustained long-term and also whether XO inhibition alters the natural history of heart failure.

We find that in a mouse model of postischemic cardiomyopathy, allopurinol delivered in the drinking water markedly enhances survival after MI and also improves global cardiac function. Investigation into the mechanistic implications revealed that oral allopurinol acts as a calcium sensitizer, increasing force generation without augmenting calcium handling. Furthermore, we show that modification of proteins by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE), evident in the myocardium of heart failure mice, is prevented in the allopurinol-treated animals. These data indicate that chronic treatment with allopurinol has a beneficial effect on the natural history of heart failure in mice in terms of contractile dysfunction and survival.

**Materials and Methods**

**Induction of MI**

SV129 male mice (n=145) were anesthetized and intubated (0.2 cm³ tidal volume/110 respirations per minute). Left anterior descending
coronary artery ligation was performed as previously described with modifications. Complete occlusion of the vessel was verified by visible blanching of the myocardium distal to the tie. After verifying that mice were breathing spontaneously after the procedure, 0.024 mg Buprenex was administered for analgesia and mice were observed until recovery was complete (usually 2 to 4 hours). After recovery, mice either received 1 mmol/L allopurinol in their drinking water or no drug by random prospective assignment. Surgeries were identical in sham mice except that the vessel was not tied-off. Mice were checked daily for survival. Experimental protocols were performed at 2 and 4 weeks after MI, except for the initial groups of mice that were observed for 60 days after MI.

**Echocardiographic Assessment of Cardiac Function**

Ventricular function was assessed 2 and 4 weeks after surgery using echocardiography (Agilent Sonos 5500, 15-MHz probe, 3-cm depth). Mice were lightly sedated with isoflurane, and wall thickness and chamber dimensions were measured using a short-axis parasternal view at the level of the papillary muscles. From M-mode tracings, fractional shortening, an indicator of chamber function, was calculated as the difference between left ventricular end diastolic dimension and left ventricular end systolic dimension and expressed as percent. Values represent the average of 5 separate measurements collected during each examination. Exact determination of wall thickness using this method is inadequate. We observed structural changes that suggested changes in anterior/posterior wall thickening, but we do not report these data because of the high degree of variability in measurement reliability.

**Measurement of Infarct Size**

At 4 weeks after surgery, mice were anesthetized and hearts were removed and weighed for determination of heart weight/body weight ratio (HW/BW). Immediately after, hearts were perfused retrogradely with Krebs–Henseleit solution with butanedione monoxime (20 mmol/L) added to attenuate cutting injury; right ventricular trabeculae were dissected and further studied. A 1-mm cross-section of the heart, at mid-papillary level, was placed in 10% formalin and stained with Masson trichrome to assess fibrosis. To determine infarct size, the circumference of the left ventricle that was fibrotic was compared with viable tissue, and a score was assigned as a percent of overall left ventricular circumference. Only muscles from mice with MI >30% were included in this study.

**Isolated Ventricular Trabeculae**

Trabeculae were dissected and then mounted between a platinum–iridium basket-shaped extension of a force transducer and a hook that mice were breathing spontaneously after the procedure, 0.024 mg Buprenex was administered for analgesia and mice were observed until recovery was complete (usually 2 to 4 hours). After recovery, mice either received 1 mmol/L allopurinol in their drinking water or no drug by random prospective assignment. Surgeries were identical in sham mice except that the vessel was not tied-off. Mice were checked daily for survival. Experimental protocols were performed at 2 and 4 weeks after MI, except for the initial groups of mice that were observed for 60 days after MI.

**Steady-State Measurements**

Muscles were tetanized as previously described. Briefly, muscles were stimulated at 14 Hz at increasing concentrations of [Ca2+]i, usually 1 to 20 mmol/L for 5 to 10 seconds until force reached a plateau. [Ca2+]i was measured simultaneously with force and calculated as described. Data points falling within a 500-nmol/L range of [Ca2+]i were binned to produce pooled values as previously described. If relaxation of the muscle after tetanization was incomplete, the protocol was terminated and that muscle excluded from analysis.

**Response to β-Adrenergic Stimulation**

A subset of muscles (generally from the same hearts subjected to the protocols described) was stimulated at 4 Hz/37.5°C and exposed to increasing concentrations of isoproterenol (10 nmol/L to 1 mmol/L) for determination of β-adrenergic responsiveness. Force was recorded when the muscle had stabilized at each concentration of isoproterenol.

**XO Activity Assay**

Xanthine–oxidase activity was determined spectrophotometrically. Frozen heart tissue was homogenized in HEPES buffer containing protease inhibitors. XO activity was measured by calculating the slope of the increase in fluorescence after adding pterin (0.010 mmol/L), which actually measures conversion of pterin to isoxanthopterin. The reaction was stopped by adding allopurinol (50 μmol/L). To calibrate the fluorescence signal, the activity of a standard concentration of isoxanthopterin was measured. Values are expressed as nmol/min per milligram of protein. Protein concentration of homogenates was determined by Lowry assay.

**Western Blot Analysis of HNE-Modified Proteins**

Left ventricular homogenates from sham (n=3), MI–control (n=4), and MI–allopurinol (n=4) were separated on a 4% to 12% gradient gel under nondenaturing conditions as previously described and using an anti-HNE antibody and electroblotted onto nitrocellulose membrane. Primary antibody binding was visualized using a horseradish peroxidase–labeled chemiluminescence assay.

**Statistical Analysis**

The data are presented as mean±SEM. Survival curves were generated using the Kaplan–Meier survival function using statistical software (STATA, v 7.0). The remainder of the data was analyzed using Student t test, ANOVA, or MANOVA, when appropriate, using SPSS statistical software. P<0.05 was considered statistically significant.

**Results**

**Improved Survival After Allopurinol Treatment**

After ligation of the left anterior descending, mice either died within 7 to 14 days after MI or developed chronic heart failure. In the weeks after MI, mice developed signs of heart failure, including tachypnea, anasarca, and lethargy. Mice treated chronically with allopurinol after MI were twice as likely to survive (39% versus 19%; n=75 allopurinol; n=63 sham) for 60 days as compared with controls (Figure 1a). We confirmed that XO activity was elevated in untreated mice 4 weeks after MI, but significantly attenuated in the allopurinol group, restoring XO levels to those of sham-operated mice (Figure 1b, inset). These findings indicate functional upregulation of XO in murine postischemic cardiomyopathy and verify that chronic oral allopurinol reduces myocardial XO activity.
Mechanism of Increased Survival

Having observed that allopurinol increased survival, we sought to determine the mechanism for this salutary effect. First, to determine whether XO suppression reduced infarct size, we examined the hearts histologically and measured HW/BW ratio. Neither infarct size (51±4, MI–control versus 42±5, MI–allopurinol) nor hypertrophy (as reported by HW/BW ratio (mg/g)=9.63±0.6 versus 9.42±0.5, control versus allopurinol) was altered by allopurinol (Figure 1c and 1d). Second, we measured cardiac function in vivo by echocardiography 2 and 4 weeks after MI. Mice treated with allopurinol exhibited a marked enhancement of cardiac systolic function 2 and 4 weeks after MI, as revealed by the significant increases in fractional shortening. There were no significant differences in heart rate in any of the groups. Consistent with the histology and HW/BW data, echocardiographic measures of wall thickness, and diastolic chamber dimensions showed no differences between groups (data not shown), (Figure 2a, 2b, and 2c).

The mechanism of benefit identified here, namely preservation of systolic function and contractility without gross structural remodeling, contrasts with that of angiotensin-converting enzyme inhibitors and other drugs that have been shown to improve survival in heart failure. Such agents given early after MI suppress chamber remodeling—the hearts become less dilated and infarct expansion is attenuated, but they do not primarily preserve contractility.19

Increased Force Generation in Heart Muscle From Allopurinol-Treated Mice

To further characterize the striking improvements in survival and cardiac function, we examined calcium cycling and contractile force in cardiac muscle from mice treated with allopurinol versus controls 4 weeks after MI. Chronic oral allopurinol leads to marked augmentation of contractile force in the failing myocardium. Force was not fully restored to the normal levels seen in sham-operated mice but was nevertheless significantly improved in the allopurinol mice relative to untreated controls (P<0.05, P<0.01, P<0.01, at 1.0, 1.5, 2.0 mmol/L [Ca^{2+}]), respectively; Figure 3a and 3b). Allopurinol treatment accelerated relaxation as exemplified in
Figure 3a; pooled data for time to 50% and 90% relaxation verified the significance of this effect ($P<0.05$, MI–control versus MI–allopurinol; ANOVA for each $[\text{Ca}^{2+}]_0$). There were no statistically significant differences in calcium transient amplitude at any $[\text{Ca}^{2+}]_i$, although there was a general trend toward decreased $[\text{Ca}^{2+}]_0$ in the allopurinol group. These data reveal that allopurinol exerts a beneficial calcium-sensitizing effect in the failing myocardium, resulting in more force generation at any given level of activator calcium, without augmenting activator calcium.

Myofilament Calcium Responsiveness

The most rigorous measure of myofilament calcium responsiveness is the steady-state relationship. We assessed this relationship by tetanization of cardiac muscle as described. There were all mice subjected to MI had a reduction in $F_{\text{max}}$ when compared with sham-operated mice ($P=0.01$), the mice treated with allopurinol exhibited a 50% ($P<0.05$) increase in maximal $\text{Ca}^{2+}$-activated force relative to untreated controls (Figure 4a). Additionally, the cooperativity of activation, as measured by the Hill coefficient, was greater in the allopurinol mice ($P<0.01$) compared with the controls, but there was no change in the midpoint of activation. In summary, allopurinol increases maximal $\text{Ca}^{2+}$-activated force and cooperativity but does not shift the range of activation (Figure 4a). Additionally, the cooperativity of activation, as measured by the Hill coefficient, was greater in the allopurinol mice ($P<0.01$) compared with the controls, but there was no change in the midpoint of activation. In summary, allopurinol increases maximal $\text{Ca}^{2+}$-activated force and cooperativity but does not shift the range of activation. Thus, unlike conventional calcium-sensitizing drugs (eg, levosimendan, sulmazole), which may exacerbate diastolic dysfunction, allopurinol does not shift myofilament sensitivity, contributing to its unique profile of action. These distinctive changes in excitation–contraction coupling rationalize the improvement in diastolic relaxation, which was evident in the MI–allopurinol group.

Improved $\beta$-Adrenergic Response

Desensitization of the $\beta$-adrenergic signaling cascade is a common manifestation of heart failure. To determine if treatment with allopurinol could potentiate the responsiveness of cardiac muscle to $\beta$-stimulation, we examined the effect of increasing concentrations of isoproterenol. In the MI mice, the response to isoproterenol was markedly blunted ($P<0.001$), as has been shown in other animal models of heart failure, but oral allopurinol restored the $\beta$-adrenergic response to near-normal levels (Figure 4b). These are the first data to our knowledge to show that chronic treatment with an XO inhibitor mediates long-term improvements in the $\beta$-adrenergic response, extending previous work showing a benefit with acute intravenous administration.

Allopurinol Prevents Lipid Peroxidation of Proteins

The reactive short-chain HNE is formed in biological tissues during oxidative stress as a result of lipid peroxidation. To determine if allopurinol prevented oxidative damage to proteins, we measured HNE-modified proteins in myocardial homogenates. Whole hearts were used because the amount of tissue necessary to conduct the experiments exceeded the yield of homogenate from the RV alone. Figure 5 shows that MI alone leads to oxidative changes in protein structure, as evidenced by an increase in the level of HNE-modified protein. Molecular weights are not shown. However, in the
allopurinol-treated mice, oxidation of proteins significantly decreased the level of HNE-modified protein as determined by Western blot. Similar results were obtained in sham-operated mice.

**Discussion**

We have shown that chronic treatment with oral allopurinol improves survival and restores cardiac contractile function in a mouse model of postischemic cardiomyopathy, most likely attributable to prevention of oxidative changes in myocardial proteins. The elevation in XO activity provides a rationale for augmentation of free radical production; however, it does not completely answer the question of whether the effect of allopurinol is directly related to blockade of XO-generated free radicals. It is conceivable that allopurinol is augmenting contractility by an XO-independent mechanism. Oxypurinol, the active metabolite of allopurinol, mimics the actions of allopurinol on cardiac muscle, hinting that the effects are attributable to XO inhibition.2,15 Nevertheless, further studies are required to elucidate the role of XO inhibition in myocardium, versus an allopurinol-specific effect or an indirect effect attributable to suppression of uric acid levels. The observation of increased XO activity in this mouse model of heart failure, which resembles human heart failure secondary to previous MI, confirms previous observations in dogs, rats, and in humans with heart failure.23,25 What is new is the finding that chronic treatment with allopurinol increases survival, augments ventricular function, reprograms excitation–contraction coupling, and suppresses oxidative protein modifications (HNE). Force is greater in allopurinol-treated hearts, without a concomitant increase in activator calcium availability, which is potentially very important to the failing human heart. An interesting observation was that force was increased in sham-operated mice administered allopurinol therapy. However, the number of mice studied was insufficient to achieve significance, and previous studies have shown little or no net effect on force in naïve myocardium.2,26 The results are not attributable to ordinary suppression of oxygen free radicals in the course of ischemia and reperfusion (during dissection of trabeculae), because animals were not pretreated with the XO inhibitor before surgery.

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**Figure 3.** Allopurinol increases twitch force without changing calcium transient amplitude. A, Representative twitches and calcium transients from sham, MI, and MI-allo ventricular trabeculae at 1.5 mmol/L [Ca\(^{2+}\)]\(_i\). F\(_{\text{dav}}\) is shown on the left; calcium transients are on the right. [Ca\(^{2+}\)]\(_i\) was calculated as described in Materials and Methods. B, Top panel shows calcium-transient amplitude with increasing [Ca\(^{2+}\)]\(_o\). Bottom panel shows twitch force development under similar conditions of [Ca\(^{2+}\)]\(_o\); (n=6 sham, n=5 MI-ctrl, and n=6 MI-allo muscles; *P<0.05).
been identified.

...and are similar to sham-operated. Modified proteins have not
untreated mice, which are abolished by allopurinol treatment
in the presence of ryanodine (1 μmol/L) at various [Ca2+]o.

Figure 5.

These findings suggest that XO, a potential mediator of
oxidative stress, is more than just a reporter of illness and also
produces oxidative changes in proteins that undermine car-
diac function. Although we have yet to identify the precise
protein targets for oxidative modifications, our working
hypothesis is that oxidation of myofilament proteins dynam-
ically changes cross-bridge cycling, which leads to increases
in mortality (early) and progression of heart failure (late).

Figure 4. Allopurinol increases maximal force and response to
β-adrenergic stimulation. A, Tetanization of ventricular trabecu-
lae in the presence of ryanodine (1 μmol/L) at various [Ca2+]o.

Data within 250 nmol/L [Ca2+]o were pooled and forces averaged
for clarity of presentation. Steady-state forces shown fitted with
the Hill equation, Fmax, nHill, and Ca50 were calculated for each
individual muscle and averaged for fitting. Black circles indicate
sham, black triangles indicate M1–allo, and black squares indi-
cate M1–ctrl. B, Dose response to increasing doses of isoproter-
enol in isolated cardiac muscle (n = 10 sham, 5 M1–ctrl, 5
M1–allo).

The present data are not only the first to implicate
oxidative stress as a contributor to mortality but also argu-
against the conventional wisdom that positively inotropic
therapies necessarily worsen outcome.27 The key to success
here may lie in the fact that allopurinol acts at the myofil-
ament level, rather than at the level of calcium cycling. An
increase of myofilament calcium responsiveness would be
expected to lower energy consumption and increase the
mechanical efficiency of contraction. Allopurinol-induced
reprogramming of excitation–contraction coupling may thus
reverse the characteristic “energy starvation” of heart fail-
ure.28 Acute administration of allopurinol markedly improves
energetics in canine and human heart failure.3,4 It will be
important to determine whether similar beneficial energetic
effects are observed with alternative long-term XO inhibitor
therapies.

Although studies in the isolated perfused rat heart have
shown that ischemia leads to lipid peroxidation of pro-
teins29–31 that can be attenuated by antioxidants,24 there were
no studies in which there is chronic inhibition of radical
production in heart failure with concomitant preservation of
protein structure. We measured HNE to demonstrate that
protein modification by oxidation reactions could be pre-
vented by treatment with allopurinol. This, by no means, is
the only or the most effective way to elucidate whether the
allopurinol effect was attributable to prevention of XO-
generated free radical damage. However, it is enough of a
preliminary observation to motivate future investigation into
this topic. Our future studies will be designed to answer the
question of how allopurinol exerts its effect and to identify
the specific proteins that are modified. It is clear that chronic
treatment with allopurinol leads to restoration of cardiac
function and that β-adrenoceptor responsiveness without
increasing activator calcium is important, but additionally
these data show that protein changes are prevented.

XO inhibitor therapy is particularly appealing as a ther-
aputic option for various reasons. First, allopurinol is a
respected drug that has been used for decades to treat gout. Its
safety record is marred by a non-negligible incidence of
allergic reactions, a limitation that may be mitigated by the
use of the active metabolite oxypurinol.32 Second, there are
already some hints in the clinical literature that XO inhibition
may be beneficial in human heart failure. Increased levels of
uric acid strongly correlate with mortality rates in congestive
heart failure;33–36 moreover, in a retrospective analysis of a
large Scottish cohort, patients using allopurinol (for gout)
exhibited lower mortality than otherwise comparable con-

controls.37 Third, the mechanism of action is unique and thus
would be expected to synergize with and potentiate the
beneficial effects of conventional agents (including angioten-
sin-converting enzyme inhibitors and β-blockers). Consistent
with this notion, mechanical efficiency was augmented by
short-term allopurinol in patients with dilated cardiomyopa-
thy, despite the fact that such patients were administered
standard heart failure regimens.3 Therefore, even though the
basal level of XO activity in the human heart and its eleva-
tion in human congestive heart failure, are controversial, allopuri-
nol nevertheless leads to improvements in cardiac function.

The most important observation in this study is the repro-
gramming of excitation–contraction coupling, such that less
calcium produces more force; this opens the tantalizing
prospect that chronic treatment with allopurinol will increase
mechanical efficiency and thereby reverse the characteristic
energy starvation of heart failure. Until now, the importance
of energy starvation, attributable to uncoupling of cardiac
mechanics from energy use in heart failure, has remained
conjectural. The present observations motivate clinical trials

Figure 5. Western blot showing free radical-derived modifica-
tions to cardiac proteins. Left ventricle homogenates were per-
formed on 4% to 12% SDS-PAGE and probed with a polyclonal
antibody to 4-hydroxy-2-nonenal (HNE) Michael adducts (20
μg/μl protein per lane). Data show lipid peroxidation products in
untreated mice, which are abolished by allopurinol treatment
and are similar to sham-operated. Modified proteins have not
been identified.
to determine whether patients will derive similar mortality and contractility benefits to those seen in mice.

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