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Effects of urate-lowering agents on arrhythmia vulnerability in post-infarcted rat hearts

Tsung-Ming Lee [a, b, c, d], Shinn-Zong Lin [e], Nen-Chung Chang [d, f, *]

[a] Department of Medicine, Cardiology Section, An Nan Hospital, China Medical University, Tainan, Taiwan, ROC
[b] Department of Medicine, China Medical University, Taichung, Taiwan, ROC
[c] Cardiovascular Research Laboratory, China Medical University Hospital, Taichung, 40447, Taiwan, ROC
[d] Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
[e] Neuropsychiatry Center, China Medical University Hospital, Taichung, Taiwan, ROC
[f] Division of Cardiology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan, ROC

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ABSTRACT

Hyperuricemia has been shown to be associated with ventricular arrhythmias. However, the mechanisms remained unknown. We assessed whether different urate-lowering agents can attenuate arrhythmias through lowering urate itself or inhibiting xanthenes oxidize (XO) activity in infarcted rats. Male Wistar rats after ligating coronary artery were randomized to either allopurinol, or febuxostat, chemically unrelated inhibitors of XO, benzbromarone or vehicle for 4 weeks. Post-infarction was associated with increased oxidant stress, as measured by myocardial superoxide, isoprostane, XO activity and dihydroethidine fluorescence staining. Measurement of myocardial norepinephrine levels revealed a significant elevation in vehicle-treated infarcted rats compared with sham-operated rats. Sympathetic hyperinnervation was blunted after administering both XO inhibitors, assessed by immunofluorescent analysis, Western blotting and real-time quantitative RT-PCR. Besides, the XO inhibitors-attenuated nerve growth factor levels were reversed in the presence of peroxynitrite generator. Arrhythmic scores in the XO inhibitors-treated infarcted rats were significantly lower than that in vehicle. For similar levels of urate lowering, the uricosuric agent benzbromarone had no beneficial effects on oxidative stress, sympathetic hyperinnervation or arrhythmia vulnerability. Chronic use of XO inhibitors, but not uricosuric agent, down-regulated sympathetic innervation probably through a superoxide-dependent pathway and plays a role in the beneficial effect on arrhythmogenic response.

1. Introduction

Hyperuricemia is independently associated with a worse prognosis in a wide cohort of patients with cardiovascular disease (1). Patients with angiographically confirmed coronary artery disease with serum urate levels in the upper quartile were five times more likely to die than those in the lowest quartile (2). Furthermore, allopurinol treatment may provide a survival benefit among patients with hyperuricemia (3). However, the debate is ongoing whether urate itself is actively involved in these processes or whether it functions merely as an indicator of xanthine oxidase (XO) activity (4). Serum urate may increase because of increased generation, decreased excretion, or a combination of the two. Therefore, this study was designed to test different effects of lowering urate agents by either XO inhibition or uricosuric treatment without XO inhibition on arrhythmia after myocardial infarction (MI), a condition of high oxidative stress and high arrhythmia-induced mortality (5,6).

XO uses xanthine and hypoxanthine as reducing substrates and yields both superoxide and H_2O_2 via 1- and 2-electron reductions of molecular oxygen (7). XO has been implicated in a variety of pathophysiological states in the cardiovascular system, such as left ventricular (LV) dysfunction after MI (5). XO expression and activity, as determined by electron spin resonance spectroscopy, were found to be markedly increased in the remote myocardium of mice

* Corresponding author. Division of Cardiology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taiwan, ROC. Tel.:+886 2 27372181x3101; fax: +886 2 23911200.
E-mail address: ncchang@tmu.edu.tw (N.-C. Chang).
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after MI (5). Growing evidence also suggests its important role for increased oxidative stress in adverse LV remodeling after MI (8). Blocking XO-generated oxygen radical accumulation has emerged as an intriguing new treatment option for preventing oxygen radical accumulation and its adverse effects on ventricular remodeling (5). However, it remained unknown whether this therapeutic approach translates into meaningful beneficial pathophysiological changes in terms of postinfarction arrhythmias.

Increased sympathetic nerve density after MI has been shown to be responsible for the occurrence of lethal arrhythmias and sudden cardiac death in humans (9). Four weeks after MI, regional increase of sympathetic nerves was commonly observed at the remote zone (10). Nerve growth factor (NGF) is a prototypic member of the neurotrophin family, members of which are critical for the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems (11). The *NGF* promoter contains activator protein-1 (12), which is subjected to redox regulation through its conserved cysteine residue (13). Previous studies have shown that peroxynitrite, the byproduct of •NO and superoxide ($O_2^{\bullet -}$), is an important trigger of NGF formation (14). A brief exposure to peroxynitrite induces *NGF* expression and secretion in astrocytes (14).

Hyperuricemia has been shown to be associated with ventricular arrhythmias (15). Up-regulated XO enzymatic activity is recognized as the key pathophysiologic feature of hyperuricemia (16). The pharmacodynamics of allopurinol are complex because XO forms 2 very different molecules, urate and free radicals. Recently, we have shown that free radicals can modulate the production of neurotrophic factor (17). Thus, the purpose of this study was 1) to investigate whether chronic administration of urate-lowering agents results in attenuated hyperinnervation of the heart and decreased arrhythmias through attenuated expression of *NGF*, and 2) to further confirm the role of free radicals in sympathetic innervation in a rat MI model by using a nonpurine selective XO inhibitor, febuxostat.

2. Materials and methods

2.1. Animals

The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1.1. Experiment 1 (in vivo)

Male normouricemic Wistar rats aged 8 weeks (200–250 g) were subjected to ligation of the anterior descending artery as previously described (18) resulting in infarction of the LV free wall. Rats were randomly assigned into either vehicle (saline), benzbromarone (10 mg/kg per day), allopurinol (50 mg/kg per day, Sigma, St. Louis, MO), or febuxostat (5 mg/kg per day, Teijin Limited, Yamaguchi, Japan). To confirm the role of sympathetic hyperinnervation in arrhythmic vulnerability, we added an infarcted group treated with carvedilol (β-adrenoceptor blocking agent: 5 mg/kg per day, Sigma; n = 7) (19). The doses of benzbromarone, allopurinol and febuxostat used in this study have been shown to effectively decrease urate levels without significantly changing blood pressure (20–22).

The drugs were started 24 h after infarction, at a time when they could produce maximum benefits (23). The study duration was designed to be 4 weeks because the majority of the myocardial remodeling process in the rat (70–80%) is complete within 3 weeks (24). The drugs were administered by daily oral gavage. Sham operated rats served as controls to exclude the possibility that the drugs themselves directly altered sympathetic innervation. In each-treated group, drugs were withdrawn about 24 h before the end of the experiments in order to eliminate their pharmacological actions.

2.1.2. Experiment 2 (ex vivo)

To further confirm the mechanism by which XO inhibitors act as antioxidants, a peroxynitrite generator (3-morpholinosydnonimine, SIN-1) was used in an *ex vivo* model. Four weeks after induction of MI by coronary ligation, infarcted rat hearts were isolated and subjected to no treatment (vehicle), allopurinol (10 μM), febuxostat (15 μM), allopurinol + SIN-1 (37 μM) and febuxostat + SIN-1. The doses of allopurinol, febuxostat and SIN-1 have been shown to be effective in modulating biological activities (25–27). In order to preclude non-specific actions to SIN-1, the relatively low concentration of SIN-1 was used. The heart was perfused as previously described (25). The drugs were infused for 30 min. At the end of the study, all hearts (n = 5 each group) were used for myocardial peroxynitrite measurement and Western analysis for NGF protein at the remote zone (>2 mm outside the infarct).

2.2. Hemodynamics and infarct size measurements

Hemodynamic parameters and infarct size were measured in anesthetized rats at the end of the study as described in detail in the Supplementary material online.

2.3. In vivo electrophysiological studies

Following arterial pressure measurement, the rats were intubated. Electrophysiological studies were performed as previously described (25). Occurrence of ventricular arrhythmias was analyzed according to an 8-point arrhythmia score. For a detailed method, please refer to the Supplementary material online.

2.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR) of NGF

Real-time quantitative RT-PCR was performed from samples obtained from the remote zone with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems) as previously described (25). For a detailed method, please refer to the Supplementary material online.

2.5. Western blot analysis of NGF

Samples were obtained from the remote zone at week 4 after infarction. Rabbit polyclonal antibodies to NGF (Chemicon, CA, USA) were used. Western blotting procedures were described previously (25). Experiments were replicated three times and results expressed as the mean value.

2.6. Immunofluorescent studies of tyrosine hydroxylase, growth associated factor 43, and neurofilament

In order to investigate the spatial distribution and quantification of sympathetic nerve fibers, analysis of immunofluorescent staining was performed on LV muscle from the remote zone. Tissues for assessing sympathetic innervation were incubated with anti-tyrosine hydroxylase (1:200; Chemicon, CA, USA), anti-growth associated protein 43 (a marker of nerve sprouting, 1:400; Chemicon, CA, USA), and anti-neurofilament antibodies (a marker of sympathetic nerves, 1:1000; Chemicon, CA, USA) in 0.5% BSA in PBS overnight at 37 °C. The analysis of the immunofluorescent staining is described in detail in the Supplementary material online.