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Transient reduction in myocardial free oxygen radical levels is involved in the improved cardiac function and structure after long-term allopurinol treatment initiated in established chronic heart failure

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KEYWORDS
Heart failure;
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Aims Oxidative stress, i.e. imbalance between reactive oxygen species (ROS) and antioxidant defences, contributes to the progression of chronic heart failure (CHF). Acute inhibition of xanthine oxidase (XO), which produces ROS, improves mechanical efficiency of the failing heart, but whether long-term XO inhibition exerts beneficial effects in CHF is unknown.

Methods and results In rats with established CHF induced by left coronary ligation, we assessed the effects of a 5-day and a 10-week treatment with the XO inhibitor allopurinol (50 mg kg^{-1} day^{-1}) on haemodynamics and left ventricular (LV) function and structure. Both acute and chronic allopurinol treatment increase cardiac output without modification of arterial pressure, but only chronic allopurinol treatment reduces LV end-diastolic pressure and LV relaxation constant. Chronic allopurinol treatment decreases both LV systolic and diastolic diameters, but acute allopurinol treatment only decreases LV systolic diameter. Moreover, chronic allopurinol decreases LV weight and collagen density. Despite XO inhibition after acute and chronic allopurinol treatment, as both treatments reduce uric acid plasma levels, only acute allopurinol treatment reduces LV ROS determined using electron spin resonance spectroscopy. However, the CHF-enhanced myocardial thiobarbituric acid reactive substances levels were never modified.

Conclusion In experimental CHF, long-term allopurinol treatment, initiated in a pathological state of overt CHF, improves LV haemodynamics and function and prevents LV remodelling. These long-term effects are, at least partially, caused by a transient reduction of myocardial ROS shortly after initiation of allopurinol treatment, but whether other mechanism(s), independent of myocardial redox 'status', such as reduced inflammation, are implicated remains to be confirmed.

Introduction

Chronic heart failure (CHF) is characterized by a progressive left ventricular (LV) remodelling,[1,2] and despite the introduction of medical treatment, has a poor prognosis.[3] Although the involvement of systemic and cardiac haemodynamics and neurohumoral factors in the progression of CHF are extensively studied and are recognized as therapeutic targets,[4–6] 'oxidative stress' may be another therapeutic target, as oxidative stress remains elevated in patients with CHF under active treatment[7,8] and thus continues to exert deleterious effect(s). Indeed, oxidative stress, i.e. imbalance between reactive oxygen species (ROS) production and antioxidant defences, is increased after myocardial infarction and/or in CHF, and is thought to induce ventricular dilation and dysfunction as well as impairment of vascular endothelial function. Furthermore, the reduction of oxidative stress by antioxidants, administered before or soon after induction of myocardial infarction or once CHF is established, exerts beneficial effects. Indeed, antioxidants, such as vitamin E,[9] DMTU,[10] and probucol[11,12] preserve LV function and attenuate LV dilation and/or collagen accumulation induced by myocardial infarction. Finally, in humans, semi-chronic administration of vitamin E alone or together with vitamin C reduces pulmonary vascular endothelial dysfunction,[13] but antioxidant supplementation modifies neither prognostic nor functional markers of CHF and quality of life.[14,15]

As antioxidants prevent the deleterious effects of ROS through 'neutralization' when the latter are produced,

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inhibition of ROS producing enzymes should also be beneficial in CHF. Xanthine oxidase (XO), for which LV tissue expression is up-regulated in CHF,[16] is prominent among ROS producing enzymes and its short-term inhibition exerts beneficial cardiac and vascular effects. Indeed, allopurinol improves myocardial efficiency by decreasing the energy cost of the left ventricle[16,17] and opposes peripheral endothelial dysfunction in patients with CHF.[8] Furthermore, high uric acid plasma levels are correlated with 'poor' survival.[18] Recently, it has been reported that allopurinol treatment initiated immediately after coronary artery ligation in mice reduces cardiac remodelling, illustrating that XO inhibition is beneficial in myocardial infarction.[19,20] However, whether long-term allopurinol, initiated once CHF is established, improves LV function and opposes LV remodelling is unknown. This study evaluates, in a rat model of CHF, the acute and long-term effects of the XO inhibitor allopurinol on systemic and cardiac haemodynamics, LV function and remodelling, and on the oxidative status of the myocardium.

Methods

The investigation conforms to 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).

Experimental protocol

Myocardial infarction was produced in 11-week-old male Wistar rats (Charles River, L'arbresle, France) by ligation of the left anterior descending coronary artery, according to the method of Pfeffer *et al.*[21] and modified in our laboratory.[22] In brief, animals were anaesthetized (ketamine and xylazine; 80 and 5 mg/kg, respectively; i.p.), intubated, and mechanically ventilated using a rodent ventilator (Apelex, Massy, France). A left thoracotomy was performed and the heart was exposed, a 6.0 polypropylene snare was passed around the proximal left coronary artery and the suture was tied. Successful occlusion was confirmed by visual cyanosis. Fifteen minutes after occlusion, the chest was closed in three layers (ribs, muscles, and skin). The animals were allowed to recover from anaesthesia. Rats were subjected to the same surgical procedure except ligation of the coronary artery served as sham group.

Interpretation of the results obtained after long-term treatment does not allow separation of the direct, i.e. acute, effects of allopurinol and indirect and long-term effects induced by the improvement of haemodynamics and/or cardiac remodelling. To avoid this experimental bias, two independent protocols were performed.

Long-term allopurinol treatment

Eight weeks after ligation, and thus after infarct healing and development of overt CHF, 30 infarcted rats were randomized in two groups either untreated or treated with allopurinol (Sigma, France) at a dose of 50 mg kg^{-1} day^{-1} as food additive for 10 weeks ($n = 15$ per group), whereas untreated sham animals were used as control. The 12 infarcted animals that died after the surgical intervention but before randomization were excluded from the study.

Short-term allopurinol treatment

Sixteen infarcted rats were randomized, 18 weeks after ligation, in two groups either untreated or treated with allopurinol at a dose of 50 mg kg^{-1} day^{-1} as food additive for 5 days (n=8 per group), whereas untreated sham animals were used as control. As for the long-term allopurinol treatment, the infarcted animals that died after the surgical intervention but before randomization were excluded from the protocol. The initiation of the short-term treatment was delayed in order to sacrifice the sham and untreated infarcted of the short- and long-term protocols at the age of 18 weeks.

Echocardiographic studies

In the chronic treatment protocol, transthoracic Doppler echocardiographic studies were performed just before and 4 and 10 weeks after starting treatment, using an echocardiographic system (HDI 5000, ATL) equipped with a 8–5 MHz transducer, as described previously.[22] Briefly, a two-dimensional short axis view of the left ventricle was obtained at the level of the papillary muscle, in order to record M-mode tracings. End-diastolic and systolic LV diameters were measured by the American Society of Echocardiology leading-edge method from at least three consecutive cardiac cycles.[23]

In addition, LV outflow velocity was measured by pulsed-wave Doppler, and cardiac output was calculated as CO = aortic VTI × [π × (LV outflow diameter/2)^2] × heart rate, where VTI is velocity-time integral.

Haemodynamic studies

At the end of the treatment, rats were anaesthetized with Brietal (50 mg kg^{-1}, i.p.), the right carotid artery cannulated with a micromanometer-tipped catheter (SPR 407, Millar Instruments, USA) and advanced into the aorta for recording of arterial blood pressure. The aortic catheter was then advanced into the left ventricle for the determination of LV pressures and its maximal and minimal rate of rise (dP/dt_{max} and dP/dt_{min}) and LV relaxation constant Tau.

Infarct size

Infarct size was determined as described previously.[22] After assessment of LV haemodynamics, atria and right and left ventricles were weighted separately, and a section of the left ventricle was immersed in fixative solution. After fixation, the sections were dehydrated and embedded in paraffin. From these sections, 5 μm thick histologic slices were obtained and were stained with Sirius Red.

For the measurement of infarct size, slices were placed under a video microscope (Microwatcher VS-30H, Mitsubishi Kasei Co-operation) with a 20-fold enlargement lens. The endocardial and epicardial circumferences of the infarcted tissue and of the left ventricle were determined using an image analysis software (Cyberview, Cervus International). Infarct size was calculated as (endocardial + epicardial circumference of the infarcted tissue)/(endocardial + epicardial circumference of the left ventricle) and expressed as a per cent.

Moreover, LV collagen density in 'viable' part of the left ventricle was determined, as previously described[22] and expressed as the surface occupied by collagen divided by the surface of the image. Perivascular collagen was excluded from this measurement.

Lipid peroxidation

The lipid peroxide content of the left ventricle was studied by determining the thiobarbituric acid reactive substances (TBARS) for the estimation of malondialdehyde (MDA) content, as previously described.[24] In brief, the myocardium was dissected, homogenized (10% w/v) in 0.2 M Tris–HCl, 0.16 M KCl, pH 7.4 supplemented with 0.02% butylated hydroxytoluene and incubated for 1 h at 37 C. After mixing, a 1.0 mL aliquot was withdrawn from the incubation mixture and placed in a Pyrex tube. This was followed by the addition of 1 mL of 40% trichloroacetic acid (TCA) and 1 mL of 0.2% thiobarbiturate sodium. Tubes were boiled for 30 min and cooled on ice. Two millilitres of 70% TCA were added and the

tubes were then centrifuged at 800 *g* for 20 min. The supernatant was assayed by measuring the absorbance at 532 nm and the concentration of MDA was calculated from MDA standards. Results were expressed in nanograms of MDA mg^{-1} tissue.

ROS production

The production of ROS was evaluated with electron spin resonance spectroscopy. Briefly, biopsies of LV tissues were collected in the viable part of the ventricle, frozen in liquid nitrogen and kept at −80°C until analysis. Thawed tissues were cut into 0.5/10 mm slices and incubated at 37°C for 1 min in 50 mM phosphate buffer containing 1 mM deferrioxamine and 1 mM 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl pyrrolidine hydrochloride (CPH, Noxygen, Hamburg, Germany). The oxidation of CPH into the paramagnetic nitroxyde $CP^{\bullet}$, driven mainly by superoxyde and peroxynitrite reactions, was measured by EPR exactly 3 min after the beginning of incubation. Tissue slices were introduced into 0.5 mm i.d. quartz capillary tubes inserted inside a TMH_{384} cavity. Spectra were recorded at room temperature with a Bruker ESP300E X-band spectrometer (Bruker, Wissembourg, France) with the following acquisition parameters: microwave power, 20 mW; microwave frequency, 9.74 GHz; modulation amplitude, 2.02 G; modulation frequency, 100 kHz; gain, 5×10^4; scan rate, 0.71 G s^{-1}; time constant, 655 ms; and conversion time, 82 ms. Intensity of the spectra was measured from the height of the central line and expressed in arbitrary units per milligram of wet tissue.

Matrix metalloproteinases

Matrix metalloproteinase (MMP)-2 gelatinolytic activity was measured in the non-infarcted left ventricle by gelatin zymography, as previously described.(25) In brief, frozen LV tissue was crushed with a mortar and pestle at liquid nitrogen temperature and then homogenized by sonication in 50 mmol/L Tris–HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. Samples were diluted with 0.5 mol/L Tris–HCl (pH = 6.8), 10% sodium dodecylsulfate (SDS), 60% saccharose, and 1% Bromophenol Blue. Twenty micrograms of total proteins and gelatinase zymography standards (Chemicon International, USA) were loaded onto electrophoretic gel (10% SDS–PAGE) containing 1 mg/mL porcine gelatin (Sigma Aldrich). The gels were run at constant 200 V through the stacking phase and separating phase, maintaining a running buffer at 4°C for 1 h, and then washed with 2.5% triton X-100 twice for 15 min under agitation at room temperature. Gels were incubated overnight at 37°C in incubation buffer containing 10 mmol/L Tris base, 45 mmol/L Tris–HCl, 0.2 mmol/L NaCl, and 5 mmol/L $CaCl_2$. Gels were stained in 35% ethanol, 10% acetic acid, and 0.2% Coomassie Blue and were bleached in 35% ethanol, 10% acetic acid. Areas of MMP digestion were visualized by negative staining and quantification of pro-MMP-2 activity was performed by densitometry analysis with beta-vision program. Pro-MMP-2 activity was confirmed on addition gels with incubation buffer containing 10 mmol/L ethylenediaminetetraacetic acid for the inhibition test and 1 mmol/L aminophenylmercuric acetate for activation test.

XO expression

Protein expression of XO was determined by western blot analysis. LV tissue was homogenized in buffer containing 0.25 M sucrose, 0.2 mM phenylmethylsulfonylfluoride, and 10 mM DL-dithiotreitol. After centrifugation (40 min at 40 000 *g*), the supernatant was equilibrated to a final concentration of total protein 1.25 mg/mL with buffer containing 62.5 mM Tris–HCl pH 6.8, 2% SDS, 0.75% sucrose, 0.01% Bromophenol Blue, and 5% β-mercaptoethanol. After heating (95°C) for 5 min, 20 μL of sample was separated by 7.5% SDS–PAGE for 1 h at 100 V and transferred to nitrocellulose membrane. To ensure equivalent quantitative transfer efficiency of proteins, the nitrocellulose membrane was stained with Ponceau solution. To prevent non-specific blotting, nitrocellulose membrane was incubated in buffer containing 6 % milk, 1× phosphate buffer saline, and 0.1 % Tween 20 for 2 h at room temperature. Nitrocellulose membrane was then incubated with Mouse monoclonal antibody anti-XO primary antibody (Interchim, 1/1000, 1 h), followed by incubated with peroxydase-conjugated second antibody Donkey anti-mouse (Jackson Immunoresearch, 1/1000, 1 h). A total of 150 kDa bands were detecting using an enhanced chemiluminescence kit (Roche, France) and exposed to hyperfilm ECL (Amersham Bioscience). Quantification was performed by density analysis with Biocom image software.

Statistical analysis

All results are given as mean ± SEM. All parameters were compared using a one-way ANOVA at each time interval, followed, in case of significance, by a two-side Tukey test for multiple comparisons. Differences between groups were considered significant at the level $P < 0.05$. This statistical design avoids the bias related to spontaneous mortality throughout the 10-week treatment period. Furthermore, since the values of the different echographic and haemodynamic parameters determined in sham and untreated CHF animals of the short- and long-term studies were not statistically different, these values were pooled.

Results

Mean infarct size in 5-day and 10-week allopurinol treated ligated group were identical (47 ± 6 and 48 ± 9%, respectively) when compared with the untreated ligated group (47 ± 10%). In the 10-week treatment protocol, two untreated ligated and one allopurinol treated ligated rat died and were excluded from statistical analysis.

Effect of left coronary artery ligation

Ten weeks after ligation, LV systolic and diastolic diameters were increased, whereas cardiac output was decreased and E and A waves impaired (*Table 1*). Moreover, LV diastolic and systolic diameters continued to increase during the last 10 weeks of the study, whereas cardiac output and LV E as well as A waves remained impaired (*Figures 2–6*). After 10 weeks, LV cavity dilation was associated with an increase in LV weight and a significant collagen accumulation in the 'viable' part of the left ventricle.

Effect of acute and chronic allopurinol treatment

Allopurinol did not modify mean arterial blood pressure after 5 days or 10 weeks of treatment, but increased cardiac output and reduced total peripheral resistance (*Figure 1*).

Furthermore, the 5-day allopurinol treatment did not modify LV end-systolic pressure, LV relaxation constant Tau, LV dP/dt_{max} and dP/dt_{min}, but it tended to reduce LV end-diastolic pressure. In contrast, the 10-week treatment significantly reduced LV end-diastolic pressure, LV relaxation constant Tau, and increased LV dP/dt_{max} and dP/dt_{min}, whereas LV end-systolic pressure was not modified (*Figure 2*).

Concerning LV remodelling, the 5-day treatment with allopurinol was without any effect on LV diastolic diameter, but the 10-week treatment prevented the progression of LV cavity dilation, illustrated by a reduced LV diastolic diameter after 10 weeks. However, both the 5-day and the

Table 1 Baseline cardiac parameters 8 weeks after coronary ligation but before initiation of treatment

	Sham ($n = 8$)	Chronic heart failure	
		Untreated ($n = 10$)	Allopurinol ($n = 13$)
LVDD (mm)	6.0 ± 0.2	9.3 ± 0.1*	9.3 ± 0.1*
LVSD (mm)	2.9 ± 0.2	8.0 ± 0.1*	8.1 ± 0.1*
Stroke volume (mL/beat)	0.36 ± 0.01	0.31 ± 0.01*	0.30 ± 0.01*
Cardiac output (mL/min)	283 ± 14	222 ± 6*	220 ± 7*
E (cm/s)	61 ± 2	78 ± 4*	79 ± 3*
A (cm/s)	56 ± 2	12 ± 5*	12 ± 6*

LVSD: left ventricular systolic diameter; LVDD: LV diastolic diameter.
*$P < 0.05$ vs. sham.

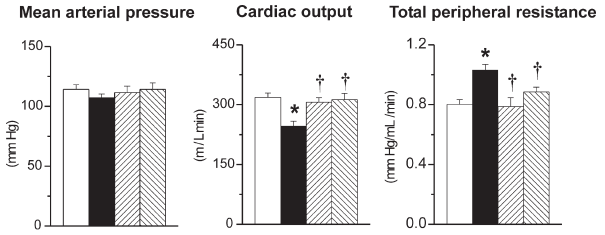

Figure 1 Mean arterial pressure, cardiac output, and total peripheral resistance determined in untreated sham (white bars; $n = 9$), untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down-hatched bars, respectively). *$P \leq 0.05$ vs. sham; †$P \leq 0.05$ vs. untreated CHF.

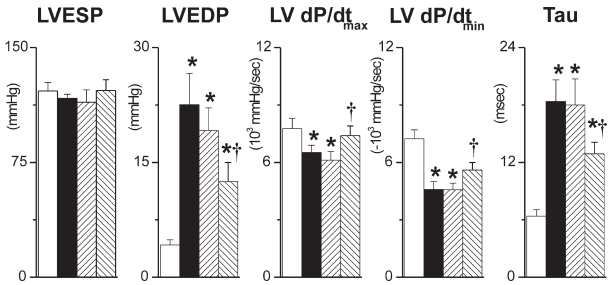

Figure 2 LV end-diastolic and end-systolic pressures, LV $dP/dt_{max/min}$ and LV relaxation constant Tau determined in untreated sham (white bars; $n = 9$), untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down-hatched bars, respectively). *$P \leq 0.05$ vs. sham; †$P \leq 0.05$ vs. untreated CHF.

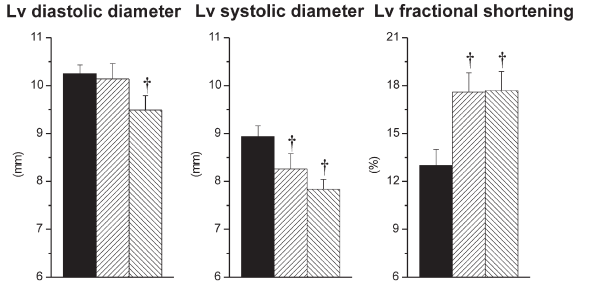

Figure 3 LV diastolic and systolic diameters and LV fractional shortening determined in untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down-hatched bars, respectively). †$P \leq 0.05$ vs. untreated CHF.

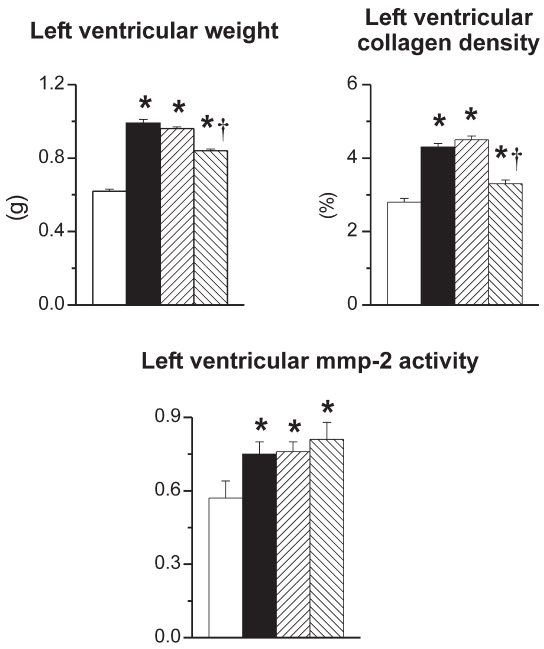

Figure 4 LV weight, collagen density and MMP-2 activity determined in untreated sham (white bars; $n = 9$), untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down-hatched bars, respectively). *$P \leq 0.05$ vs. sham; †$P \leq 0.05$ vs. untreated CHF.

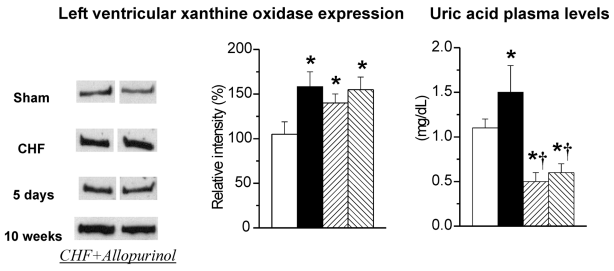

Figure 5 Western blots of LV XO expression and uric acid plasma levels determined in untreated sham (white bars; $n = 9$), untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down-hatched bars, respectively). *$P \leq 0.05$ vs. sham; †$P \leq 0.05$ vs. untreated CHF.

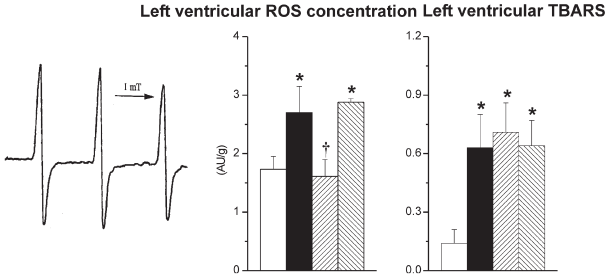

Figure 6 A typically spectrum obtained sham animals by electron spin resonance spectroscopy and LV ROS concentration as well as TBARS levels determined in untreated sham (white bars; $n = 9$), untreated CHF (black bars; $n = 16$) and 5-day ($n = 9$) or 10-week ($n = 12$) allopurinol-treated CHF rats (up-hatched and down hatched bars, respectively). *$P \leq 0.05$ vs. sham; †$P \leq 0.05$ vs. untreated CHF.

10-week allopurinol treatments reduced LV systolic diameter, resulting in an improvement of left fractional shortening after both 5 days and 10 weeks (*Figure 3*).

Moreover, chronic allopurinol treatment significantly reduced LV weight and collagen density. Furthermore, MMP-2 activity was not modified by a 5-day or a 10-week allopurinol treatment (*Figure 4*).

Concerning XO expression, CHF increased LV XO protein levels and uric acid plasma levels. Although neither 5-day nor 10-week treatment with allopurinol modified LV XO protein levels, plasma uric acid levels were significantly reduced after both 5 days and 10 weeks of treatment (*Figure 5*).

Although the increased levels of LV TBARS observed in CHF were never modified by allopurinol treatment, LV ROS levels were reduced after the 5-day but not after the 10-week treatment (*Figure 6*).

Discussion

Our results show that long-term treatment with XO inhibitor allopurinol initiated once chronic heart failure is established, improves cardiac haemodynamics, reduces LV dilation, hypertrophy and collagen accumulation, and thus ameliorates cardiac systolic and diastolic function. These long-term effects may be related to a transitory reduction of myocardial ROS production shortly after initiation of the chronic treatment and/or diminished myocardial inflammatory response.

This is, to our knowledge, the first study evaluating, the long-term effects of allopurinol initiated in a context of established overt CHF. Although recent studies revealed beneficial effects of allopurinol when treatment is started immediately after induction of myocardial infarction,[19,20] the delay of 10 weeks between the coronary artery ligation and the start of the allopurinol treatment in this study resulted not only in marked reduction of cardiac output and impairment of LV contractility and relaxation but also in marked LV cavity enlargement at the initiation of treatment. Furthermore, previous studies have reported that at this time point severe impairment of cardiac haemodynamics, enhanced cardiac collagen accumulation, pulmonary congestion, and an augmented oxidative stress resulting in an altered redox status have developed.[2,26–30]

Concerning systemic and cardiac haemodynamics, both short-term and long-term allopurinol treatment induces a peripheral vasodilatation illustrated by the reduction of total peripheral resistance, since, in the absence of any modification of arterial blood pressure, cardiac output is increased. This peripheral vasodilatation, observed after both the 5-day and the 10-week treatment and by others,[8] facilitates LV ejection and thus contributes to the increase in cardiac output. Moreover, in the case of a 10-week treatment the reduced activity of vasoconstrictor systems, such as sympathetic nervous, renin–angiotensin–aldosterone, and endothelin systems, owing to an improvement of the haemodynamics and/or cardiac remodelling could also contribute in the vascular effects of allopurinol. Indeed, such a significant improvement of the severity of CHF in our experiments after long-term allopurinol is illustrated by the reduction of LV end-diastolic pressure, Tau, and increased LV dP/dt_{max} and dP/dt_{min}.

In a context of initiating treatment in well-established CHF, both short-term and long-term XO inhibition improves systolic cardiac function, but only after long-term treatment, this is associated with an improvement of LV haemodynamics, diastolic function, and a reduction of cardiac remodelling. We can only speculate upon the mechanism(s) implicated in the acute and long-term myocardial effects observed, but the acute effects of allopurinol in CHF impaired myocardial O_2 consumption and mechano-energetic efficiency could initiate a cascade of events at the origin of these long-term effects. Indeed, CHF is characterized by chronic myocardial ischaemia, due to myocardial hypoperfusion and increased myocardial O_2 consumption, which activates local neurohumoral systems, such as angiotensin-converting enzyme, ECE, and MMPs, all implicated in the progressive deterioration of LV dysfunction, haemodynamics, and remodelling. Allopurinol opposes this vicious circle, since acute administration of allopurinol reduces myocardial oxygen consumption and improves mechanoenergetic efficiency.[16,17] Furthermore, by facilitating LV ejection and reducing LV myocardial work/O_2 demand, the vasodilatation, i.e. improvement of peripheral endothelium-dependent dilatator tone,[8,13,31] also delays the appearance of the deleterious activation of neurohumoral systems. Finally, an improvement of pulmonary circulation could also contribute to the observed effects of allopurinol on cardiac remodelling. Indeed, in rats subjected to chronic hypoxia, allopurinol prevents the development of right ventricular hypertrophy and the pulmonary hypertension,[32] thus the reduction of the right ventricular weight by allopurinol in our experiments probably reflects a major improvement of pulmonary circulation.

In parallel with the haemodynamic effects, long-term inhibition of XO by allopurinol reduces LV dilation and hypertrophy. Systemic and cardiac haemodynamic unloading is probably involved, through reduced neurohumoral activation, in this anti-remodelling effect. Simultaneously with a reduction of cardiac hypertrophy, allopurinol reduces LV collagen accumulation, resulting from a reduced synthesis whereas extracellular matrix turnover remains elevated. Indeed, collagen degradation remains elevated as MMP-2 gelatinase activity is not modified by allopurinol, and this, together with the reduction of LV collagen density, suggests that, although collagen synthesis has not been determined, allopurinol reduces, directly or indirectly, collagen synthesis. It must be stressed that whatever the mechanism(s) involved, the reductions of LV dilation, hypertrophy, and collagen are beneficial and involved in the improvement of both systolic and diastolic cardiac function observed after long-term allopurinol treatment.

With regard to the cellular mechanism(s) involved, the transient reduction in the production of ROS owing to the inhibition of XO and thus reduction of cardiac 'oxidative stress' is probably at the origin of the beneficial effects of allopurinol. Indeed, despite the increase of expression of the myocardial XO, ROS production is reduced after 5 days of treatment, but not after 10 weeks. The reduction of ROS production shortly after XO inhibition may initiates a cascade of events at cardiac and vascular levels. Indeed, the progression of CHF is associated with an enhanced expression and/or expression of pro-oxidant enzymes, among which XO, NADPH oxidase, and mitochondrial respiratory chain leading to an enhanced

free radicals production. Normally, these free radicals are inactivated by either endogenous free radical scavengers, such as vitamin A or vitamin E, or by antioxidative enzymes, such as SOD or catalase. However, when endogenous antioxidant reserve and antioxidant enzyme activities are/become exhausted,[9] free radicals start to accumulate and damage cellular proteins and DNA, inactivate (endothelial) NO, and provoke LV mechanoenergetic uncoupling, resulting, on the long term, in cardiac and vascular dysfunction and/or remodelling.

Finally, besides the transient reduction of ROS production, reduction of local inflammation related to a reduction of myocardial uric acid levels *per se* or ROS levels could be another complementary mechanism involved in the beneficial effects of allopurinol. Indeed, the enhanced myocardial tissue levels of uric acid observed in CHF[33] might lead to the formation, within the myocardium, of monosodium urate microcrystals, which provoke the activation of inflammatory processes, i.e. the secretion of TNFα.[34] Although such a mechanism remains highly speculative, it should be stressed that in patients with CHF, serum uric acid concentration correlates with markers of chronic inflammation[35,36] and thus the reduction of plasma uric acid concentration suggests a reduction in inflammation.

It must be stressed that after 10 weeks of treatment myocardial ROS production determined by electron spin resonance in the allopurinol group are similar to those observed in untreated CHF animals, suggesting that at this particular time point myocardial 'oxidative stress' is not reduced. Moreover, LV TBARS levels, resulting from the accumulation of membrane lipid oxidation that occurred during the timespan preceding assessment, are not modified after 5 days and 10 weeks of treatment. Thus suggests that after an initial reduction of ROS owing to XO inhibition, the production of ROS by other pathways, including allopurinol treatment itself, since conversion of allopurinol to its active metabolite oxypurinol generates ROS, continues to increase and eventually compensate completely the reduction of XO related ROS production. However, this would mean that the long-term beneficial myocardial haemodynamic and structural effects of allopurinol in our study could be attenuated during a longer treatment period, as with other antioxidant treatments observed in humans.[15] Indeed, antioxidant supplementation modifies neither prognostic nor functional markers of CHF and quality of life, thus it remains highly speculative whether the beneficial effects of XO inhibition are associated with an improvement of morbidity and mortality in patients with CHF, but this is assessed by the ongoing clinical trial OPT-CHF.[37]

In conclusion, our results obtained in a rat model of CHF, show that long-term XO inhibition by allopurinol initiated in a context of well-established CHF improves cardiac haemodynamics as well as function and prevents LV remodelling, suggesting that XO inhibition might be a therapeutic target in the treatment of CHF. These long-term beneficial effects of allopurinol are, at least partially, due to transient reduction of myocardial ROS production shortly after initiation of treatment, but the involvement of other mechanism(s) independent of myocardial redox status, such as reduction in inflammation, remains to be determined.

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