Age-Dependent Increase in Xanthine Oxidoreductase Differs in Various Heart Cell Types

Bob Schoutsen and Jan Willem de Jong

Myocardial xanthine oxidase has been associated with reoxygenation injury induced by oxygen radicals. The damage due to myocardial ischemia and reperfusion increases with age; therefore, one would expect to find more xanthine oxidase in adult than in young hearts. Consequently, we studied the age-dependence of xanthine oxidoreductase activity in hearts, in addition to the localization of the enzyme in cultured rat-heart cells. We measured xanthine oxidase plus dehydrogenase activity in homogenates of hearts and in homogenates of cultured neonatal myocytes and nonmuscular cells. In rat heart homogenates, xanthine oxidoreductase increased from 0.5 ± 0.1 mU/g wet wt (newborn, mean ± SD) to 25 ± 4 mU/g (age 15 weeks, p < 0.001). The value for adult rabbit heart was more than 1,000 times lower and hardly detectable. Therefore, we did not study young rabbit hearts. In rat myocyte cultures, xanthine oxidoreductase activity increased from 4.2 ± 1.6 mU/g protein (2nd day of culture) to 17 ± 4 mU/g (4th day, p < 0.005). The activity in nonmuscular cells increased much more, from 10.1 ± 1.1 to 117 ± 25 mU/g (p < 0.002). The age-related increase of xanthine oxidoreductase activity in rat heart is in agreement with the implied role in reperfusion damage by the enzyme. Whether myocytes, in which the enzyme has a low activity, could be damaged in this way, remains to be studied. (Circulation Research 1987;61:604–607)

Myocardial tissue damage develops during reoxygenation after a hypoxic period and is at least partly caused by oxygen radicals. The research on oxygen-radical-mediated injury presently focuses on xanthine oxidoreductase. The mammalian enzyme exists in two configurations: the native form, xanthine dehydrogenase (EC 1.2.3.37), and its converted form, xanthine oxidase (EC 1.2.3.2). The latter generates oxygen radicals. The location of xanthine oxidase—as a possible inducer of reperfusion injury to muscular cells—is important. Hearts of young rats are less sensitive to hypoxic insult and reperfusion than hearts of adult rats. If xanthine oxidase is responsible for reperfusion injury, the xanthine oxidoreductase activity would be expected to be higher in the adult heart. Such an age-related increase of xanthine oxidoreductase has been reported for rat liver, but to our knowledge this phenomenon has not been described for heart.

We studied the relation between animal age and xanthine oxidoreductase activity in homogenates of rat-heart ventricles. In addition, we measured the enzyme in cultured myocytes and nonmuscular cells from neonatal heart.

Materials and Methods

Reagents

Nutrition mixture F-10 was produced by Flow Laboratories, Irvine, UK; sera were obtained from Boehringer, Mannheim, FRG; [8-14C]xanthine was bought from ICN Biochemicals, Inc., Cambridge, Mass.; and bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, Mo. All other products were of analytical grade.

Heart Cell Culture

Pregnant Wistar rats (T.N.O., Zeist, The Netherlands) had free access to food and water. Two days after birth, heart cells from the neonates were isolated and cultured according to the method of Link et al. The myocytes were separated from nonmuscular (F) cells with a differential attachment technique according to Blondel et al and seeded in 3.5-cm Petri dishes (Greiner, Nürtlingen, FRG). When the cells had been cultured for either 2 or 4 days, the dishes were washed 3 times with 0.9% NaCl. With phosphate buffer (30 mM K2HPO4, 0.4 mM EDTA, pH 8.3), the cells were scraped from the dishes with a rubber policeman. Cells of 15–20 dishes were pooled. Using ice-cooling, these suspensions were twice sonicated (M2/70 sonicator, MSE, Crawley, UK) for 10 seconds and stored in liquid nitrogen until they were assayed.
Heart Homogenates

Wistar rats (T.N.O., Zeist, The Netherlands) of either sex and various ages, and female New Zealand white rabbits weighing about 2.5 kg (Brockman Inst., Stiphout, The Netherlands) were used. The animals had free access to food and water. Rats three weeks and older were sedated intraperitoneally with Nembutal; younger rats were decapitated. The rabbits were stunned by a blow on the neck. Ventricles of neonatal rats from one nest were pooled (12–16 animals), as were those of three-week-old rats (4 animals). Ventricles of the older rats and the rabbits were not pooled. After excision of the hearts, blood was removed with 0.9% NaCl. The ventricles were stored in liquid nitrogen until xanthine oxidoreductase was assayed.

Xanthine Oxidoreductase Assay

Hearts and cells were homogenized in the phosphate buffer mentioned above, with a micro-dismembrator (Braun, Melsungen, FRG) cooled with liquid nitrogen. The homogenates were thawed, shaken, and centrifuged. To remove endogenous substrates and inhibitors, the supernatant was passed through a Sephadex PD10 column (Pharmacia, Uppsala, Sweden), equilibrated, and eluted with the phosphate buffer mentioned previously. They were then assayed in duplicate at 30°C for xanthine oxidoreductase (xanthine dehydrogenase plus xanthine oxidase) activity. The enzymatic HPLC method of Schoutsen et al15 was used with [14C]xanthine (2 Ci/mol). The xanthine oxidase activity was determined without nicotinamide adenine dinucleotide (NAD+), the oxidase plus dehydrogenase activity in the presence of NAD+. One unit of enzyme activity corresponds to the formation of 1 µmol urate/min.

Protein Assay

Protein was estimated according to Bradford14 with a commercially available dye mixture (BioRad, Richmond, Calif.), using bovine serum albumin as the standard.

Statistics

Regression analysis was carried out on the data, obtained with ventricular homogenates. Cell culture data were analyzed with Student’s t test. A probability value less than 0.05 was considered a statistically significant difference. Results are expressed as mean ± standard deviation (SD).

Results

Figure 1 shows the relation between age and xanthine oxidoreductase activity. The activity increased from 0.5 ± 0.1 mU/g wet wt shorty (<3 hours) after birth, to an activity of 25 ± 4 mU/g wet wt at 15 weeks of age. With a conversion factor of 39 (determined in 10 experiments), these values were 20 and 975 mU/g protein, respectively. We found that the xanthine oxidoreductase activity was about 30% lower in female than in male hearts, but the difference was not statistically significant. Therefore, Figure 1 shows pooled data obtained from animals of either sex. For every data point, about the same numbers of female and male rats were used. Xanthine oxidase was 27 ± 9% (n = 18) of the xanthine dehydrogenase activity and was independent of age.

In heart homogenates of rabbits (age about 6 months), xanthine oxidoreductase activity (3 ± 3 µU/g wet wt; n = 6) was hardly detected. This is more than 1,000 times lower than the activity in adult rat hearts, which means that rat heart is practically devoid of the enzyme. Therefore, young rabbit hearts were not investigated.

To study the xanthine oxidoreductase activity at the cellular level, cultures of neonatal heart cells were separated into myocytes and F cells (a mixture of endothelial cells and fibroblasts; Blondel et al18). The activity in the myocyte fraction on the 2nd day of culture was extremely low at 4.2 ± 1.6 mU/g protein (Figure 2), which is about 17% of the specific activity in homogenates of neonatal rat ventricles. The value for the F cell fraction was 10.1 ± 1.1 mU/g, which is 40% of the activity in neonatal ventricle homogenates. Addition of 250 µM allopurinol inhibited the reaction. After two more days of culture, the xanthine oxidoreductase activity had increased about 4 times in the myocytes, while in the F cells the increase was more than tenfold (Figure 2).

Giemsa staining revealed the purity of the myocyte fraction to be about 80%; the F cell preparations contained less than 10% myocytes. We calculated that the xanthine oxidoreductase activity in F cells and myocytes was about 11 and 2.5 mU/g protein, respectively.

Discussion

We found an age-related increase of xanthine oxidoreductase activity in rat hearts; in addition, we noted sex-dependent differences (statistically nonsignificant). These phenomena are also known for rat liver.6,12 A rise in number of capillaries in rat heart, as reported by Rakusan and Turek,19 could explain most of the
Enzyme activity was assayed as mentioned in the legend to Figure 1. Means±SD, n=3. The differences in values found in various cell cultures were statistically significant (p<0.01), as were the increases in values during culture (p<0.005).

Age-related increase of the enzyme's activity. This correlates with a higher sensitivity to reoxygenation injury in adult rat heart. In that study, smaller urate production and better ATP preservation by neonatal, reperfused rat heart was observed. Although aging rat heart also becomes more sensitive to reoxygenation injury, this cannot be attributed to xanthine oxidoreductase. The enzyme is virtually absent in male and female (this study) rabbit heart.

The xanthine oxidase/dehydrogenase ratio in heart homogenates was 0.27 and did not vary with age. The ratio may be lower in vivo, as the dehydrogenase form converts to the oxidase form during homogenization.

Kmetec et al assumed that myocytes as well as mesenchymal cells (F cells, a mixture of endothelial cells and fibroblasts) were devoid of xanthine oxidoreductase. However, we found a low specific activity of the enzyme in myocytes and a higher one in F cells. The latter was of the same order of magnitude as that found in ventricular homogenates. The higher activity in F cells, as compared to myocytes, correlates with a greater influence of allopurinol on the uptake of hypoxanthine and the energy charge in the former. Jarasch et al used histochemical techniques to demonstrate that in bovine heart, xanthine oxidase is not primarily responsible for reoxygenation damage to myocytes because the enzyme is virtually absent in rabbit heart. Rabbit heart may serve as a control devoid of xanthine oxidoreductase for studies on free-radical-induced reperfusion injury. Other species show considerable myocardial xanthine oxidoreductase activity (for review, see Schoutsen et al).

In conclusion, rat-heart xanthine oxidoreductase activity increases with age. In view of its localization and activity, the enzyme probably causes reoxygenation injury mainly to endothelial cells. Whether the enzyme plays a direct role in myocardial reperfusion damage to myocytes requires further study.

Acknowledgments

The authors wish to thank Ms. E. Keijzer for technical assistance, Dr. J.G.P. Tijssen, biostatistician, for advice, and Ms. M.H.A. Zweserijn for secretarial help.

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**KEY WORDS** • xanthine oxidoreductase • heart • heart cell • localization • development
Age-dependent increase in xanthine oxidoreductase differs in various heart cell types.
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Circ Res. 1987;61:604-607
doi: 10.1161/01.RES.61.4.604

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/61/4/604

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