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Induction of mitochondrial xanthine oxidase activity during apoptosis in the rat mammary gland

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1. ABSTRACT

Oxidative stress is an important signal for apoptosis to start. So far the mitochondrial respiratory chain has been considered as the major, if not the only, cause of such stress. Here we report that this is not the case. Xanthine oxidase, a O_2^- and H_2O_2 generating enzyme which is important in causing significant oxidative stress in the cytosol, is also present in the mitochondrial fraction of rat mammary gland. After weaning, during the involution of the mammary gland, massive apoptosis occurs. Mitochondrial xanthine oxidase activity increases and high mitochondrial H_2O_2 production takes place. Inhibition of xanthine oxidase activity by allopurinol, a specific inhibitor of xanthine oxidase activity, slows down the involution of the mammary gland due to the decrease in the number of apoptotic cells and prevents the production of H_2O_2 that occurs during apoptosis. Thus, mitochondrial xanthine oxidase by means of its production of O_2^- and H_2O_2 can maintain the apoptotic machinery during the involution of the mammary gland after weaning and could be considered necessary to maintain the apoptotic cascade during the physiological involution of tissues. Oxidative stress generated during apoptosis by mitochondria is not only due to the respiratory chain.

2. INTRODUCTION

Apoptosis is the most important way for physiological cell death. This process regulates the number of cells within tissues. Different factors can alter the careful balance of cell life and death, resulting in diverse pathological conditions (1,2). Oxidative stress associated with the mitochondrial respiratory chain has been postulated as an early event in the apoptotic process, causing early oxidation of GSH and mtDNA and increasing hydrogen peroxide (H_2O_2) production (3). This scenario points to a fine-tuned redox regulatory mechanism in mitochondria, that can result in an oxidation of the GSSG/GSH redox pair and in activation/inactivation of enzymes, leading ultimately to the liberation of pro-apoptotic compounds. However, little is known about other sources of superoxide (O_2^-) and H_2O_2 different from the mitochondrial respiratory chain. In an attempt to find alternative pathways leading to oxidative stress and apoptosis, we report here an important regulatory role of mitochondrial xanthine oxidase during the involution of the rat lactating mammary gland. We have found that this mostly cytoplasmic enzyme is also located in mitochondria and can maintain the apoptotic mechanism by its production of O_2^- and H_2O_2. Taken together, these results

show that the oxidative stress that is generated by mitochondrial xanthine oxidase can maintain apoptosis thanks to the production of a sustained oxidative stress and damage.

3. MATERIALS AND METHODS

3.1. Animals

Six month-old female Wistar rats had free access to water, were fed *ad libitum* and kept on a 12h-dark/12h-light cycle. The rats were anesthetized with sodium pentobarbital (50 mg/kg body wt. ip.). The lactating mammary gland is a well-known model of apoptosis for *in vivo* studies (4). Mammary glands were removed at the peak of lactation (day 14) from rats whose pups were removed from the mother 12, 24 and 48 hours before performing the experiment (day 12 of lactation).

3.2. Determination of apoptosis

Apoptosis was assessed by the standard kit for *in situ* quantification of apoptosis: "ApopDETEK" (ENZO). This is a method for detecting the early stages of chromosome breakdown, based on the incorporation of BIO-16-dUTP by a terminal deoxynucleotide transferase on the 3'-OH termini produced by the endonucleolytic activity associated with the apoptotic process. Apoptosis was also determined by gel electrophoresis following the procedure of Strange et al. (5) Briefly, –80^{o}C frozen tissues were grinded and incubated for 4 hours at 56^{o}C, washed and the DNA was extracted. A 3% agarose gel was used and 5µg of DNA were loaded in each well.

3.3. Allopurinol administration

We administered the drug both orally and intraperitoneally. Part of the dose was administered with drinking water (0.4 $\pm$ 0.1 mg/g of body wt.) for three days. Then, 2 hours before sacrifice the animal were injected (i.p.) with 1.6 mg/kg body wt.

3.4. Measurement of xanthine oxidase activity

Xanthine oxidase was measured as previously described (6). Briefly, isoxantopterine formation from pterine was followed by fluorimetry (excitation at 345 nm and emission at 390 nm). Although isoxantopterine is not a physiological substrate for xanthine oxidase its sensitivity is higher (6). This technique is time consuming but is more sensitive than the spectrophotometric assays.

3.5. Isolation of mitochondria

After killing the animals, their mammary glands were quickly removed. Isolation of mitochondria was performed using a standard differential centrifugation procedure as described by Rickwood *et al.* (7).

3.6. Hydrogen peroxide production in isolated mitochondria

The rate of H_2O_2 production was determined in isolated mitochondria using a modification of the method described by Barja (8). Briefly, mitochondria were incubated at 37°C with 10 mM xanthine or xanthine and 100 µM allopurinol in 2 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCL, 30 mM Hepes, 0.1 mM homovanilic acid, and 6 U/ml horseradish peroxidase. The incubation was stopped at 5, 10, and 15 min with 1 ml of cold 2 M glycine buffer containing 50 mM EDTA and 2.2 M NaOH. The fluorescence of supernatants was measured using 312 nm as excitation wavelength and 420 nm as emission wavelength. The rate of H_2O_2 production was calculated using a standard curve of H_2O_2.

3.7. Expression of proteins

Expression of proteins was detected by western blotting using the Protoblot Western Blot AP System (Biorad, Spain). XO antibodies were from Chemicon International, Inc. alpha-tubuline from Santa Cruz Biotechnology and 3-nitrotirosine antibodies were obtained from HyCult Biotechnology B.V.

3.8. Statistics

Results are expressed as mean ± SD. Statistical analyses were performed by the least-significant difference test, which consists of two steps: first an analysis of variance was performed. The null hypothesis was accepted for all numbers of those set, in which F was non-significant at the level of $P<0.05$. Second, the sets of data in which F was significant were examined by the modified t test using $p< 0.05$ as the critical limit.

4. RESULTS

4.1. Induction of apoptosis by weaning increases mitochondrial xanthine oxidase activity

Weaning induces massive apoptosis and oxidation of mitochondrial glutathione in rat mammary gland (3) in a time dependent manner, reaching a maximum 24 hours after removal of the pups. In this study we report the expression of xanthine oxidase in isolated mitochondria in weaned and in lactating mammary gland.

Figure 1 shows the presence of xanthine oxidase in mitochondrial extracts from both lactating and weaned mammary glands. Expression of xanthine oxidase increases (75% over the controls) 12 and 24 h after pup removal and returns to control values two days after weaning. Obviously the increase of xanthine oxidase activity in mitochondria isolated from the mammary gland could be due in part to contamination from the cytoplasmic enzyme. To exclude this possibility, the expression of alpha-tubuline (54 kDa) was determined in parallel as a cytoplasmic marker. Figure 2 shows that cytoplasmic contamination of mitochondria from cytoplasm was negligible. As expected, the samples from the cytoplasm showed high alpha-tubuline levels. Those from the mitochondrial fraction were free of alpha-tubuline. Therefore the xanthine oxidase activity that was found does not seem to be due to cytosolic contamination.

Figure 3 shows the xanthine oxidase activity of isolated mitochondria from lactating and 24-hour weaned mammary glands. Xanthine oxidase activity increases 150% in the mitochondria from weaned rats.

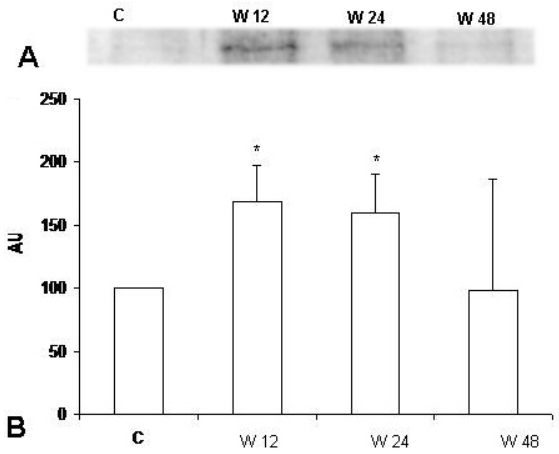

Figure 1. Presence of xanthine oxidase in mitochondria isolated from rat mammary gland. A) Representative experiment of xanthine oxidase expression in samples from isolated mitochondria from control (C) and 12, 24 and 48 hours weaned mammary glands (W 12, W 24 and W 48). B) Densitometry of xanthine oxidase expression in mitochondria from control (C) and 12, 24 and 48 hours weaned mammary glands (W 12, W 24 and W 48). Results are mean ± SD from 3 different experiments. Control is considered 100% of band intensity.

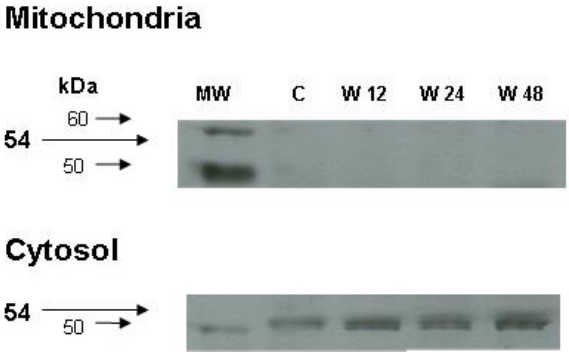

Figure 2. Expression of alpha-tubuline in mitochondria (A) and cytosol (B) of rat mammary gland. Representative experiment of alpha-tubuline (54 kDa) expression in samples from control and 12, 24 and 48 hour weaned mammary glands (W 12, W 24 and W 48). B). MW: molecular weight.

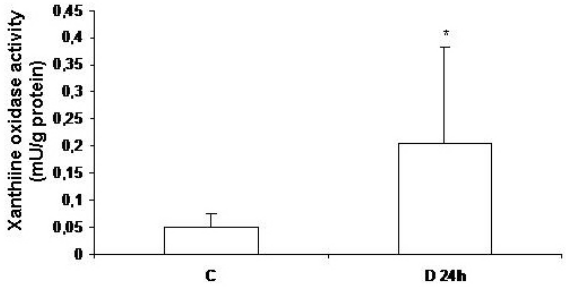

Figure 3. Xanthine oxidase activity in mitochondria from rat mammary gland. Results are mU/g protein, as mean ± SD of 5 different experiments of rat mammary gland mitochondria from control and 24-hour-weaned rats. Statistical difference is expressed as * $p<0.05$ from the control group.

4.2. Nitrated proteins in mammary gland mitochondria

Figure 4 shows a Western blot of nitrated proteins originating in mitochondria from lactating and 24 hours after weaned mammary glands. There is an increase in nitrate proteins of molecular weights between 10-20 kDa and 20-30 kDa. Xanthine oxidase has been reported as an enzyme that generates nitric oxide (9) and therefore, this fact can subsequently produce an increase in the content of nitrated proteins.

4.3. Effect of allopurinol on the activity of mammary gland mitochondrial xanthine oxidase

Isolated mitochondria were incubated for 40 min at 37ºC with 50 and 100 miicroM allopurinol, the specific inhibitor of xanthine oxidase. Both concentrations were capable of inhibiting the enzymatic activity as it is shown in Table 1. Therefore, allopurinol is able to decrease mitochondrial xanthine oxidase activity from weaned glands to only 15% with 50 microM allopurinol and to less than 10% with 100 microM allopurinol.

4.4. Hydrogen peroxide production of mitochondrial xanthine oxidase from weaned mammary glands

Several authors showed that ROS from mitochondrial origin can cause damage to DNA at the mitochondrial level, correlating it with the process of ageing (10-12) and with the physiological process of apoptosis (13). In order to determine the xanthine oxidase production of H_2O_2, mammary gland mitochondria were incubated with 10 mM xanthine, the physiological substrate of the enzyme. Allopurinol was able to inhibit mitochondrial H_2O_2 production significantly (Figure 5), reaching a levels similar to the one of lactating rats. Thus, in our model in which we used mitochondria isolated from lactating and 24-hour weaned mammary glands, mitochondrial xanthine oxidase was a significant source of H_2O_2 during mammary involution.

5. DISCUSSION

5.1. A new location for xanthine oxidase: the mitochondria

The results shown here indicate that xanthine oxidase activity exists in mitochondria and can be inhibited by allopurinol. This means that the production of ROS by mitochondria occurs due to not only the mitochondrial respiratory chain during the induction of apoptosis, but also by the catalytic activity of xanthine oxidase.

Mitochondria are key factors in the intrinsic pathway of apoptosis. During apoptosis mitochondria undergo several changes: loss of mitochondrial membrane potential, liberation of caspase activators (like cytochrome c), changes in the electron transport, alteration of the oxido-reduction equilibrium in cells, and participation of the pro- and antiapoptotic protein Bcl-2 (14). Leakage of cytochrome c from mitochondria is essential to form the apoptosome and thus to initiate the activation cascade of caspases, which subsequently results in the digestion of the genomic DNA. Moreover, mitochondria are the origin of free radicals and a fundamental source of oxidative stress in diverse cell lines. In 1999, we reported that oxidative stress

Table 1. Xanthine oxidase activity in mammary gland mitochondria from lactating and 24-hour-weaned rats incubated with allopurinol

Allopurinol (microM)	Xanthine oxidase activity in Lactating glands (microU/mg proteins)	Xanthine oxidase activity in Weaned rats (microU/mg proteins)
0	38 ± 16	193 ± 37 [1]
50	20 ± 1 [2]	4 ± 2 [4]
100	7 ± 5 [3]	3 ± 2 [5]

Statistical difference is expressed as [1] $p < 0.0005$ weaned versus lactating; [2] $p < 0.05$ lactating with allopurinol versus lactating; [3] $p < 0.005$ lactating with allopurinol versus lactating; [4] $p < 0.005$ weaned with allopurinol versus weaned; [5] $p < 0.0005$ weaned with allopurinol versus weaned.

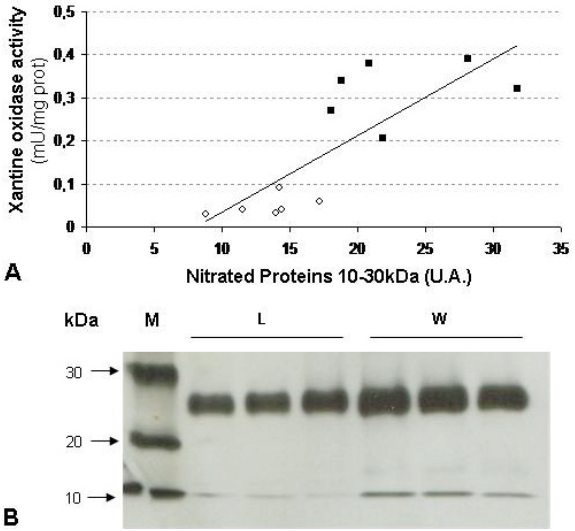

Figure 4. Nitration of low molecular weight proteins after weaning. A) Correlation between xanthine oxidase activity and low molecular weight nitrate proteins. Each point represents a different experiment. A.U. arbitrary units. B) Representative experiment of nitrated proteins in mammary gland mitochondria from control and weaned rats. M: molecular weight marker. L: lactating , W: weaned.

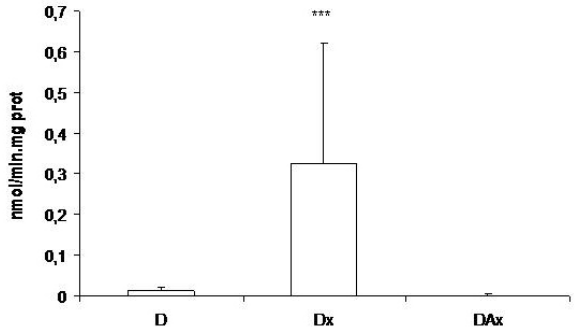

Figure 5. Hydrogen peroxide production with xanthine as substrate from mammary gland mitochondria from 24-hour-weaned rats with or without allopurinol. Results are mean ± SD of 4 different experiments. W: weaned, WX: weaned incubated with xanthine 10mM. WX+A: weaned incubated with xanthine and allopurinol 100 μM *** $p < 0.001$.

was an initial event in the apoptotic process and that the GSSG/GSH ratio increased before morphologic changes occurred in the mammary gland (3). In that same work and in others (15) it was shown that mitochondria produce oxidative stress in the initial phases of the apoptotic process in different experimental models.

Xanthine oxidase is a Mo-metalloprotein with Fe-S centers. The reaction has a broad spectrum of substrates, xanthine, hypoxanthine and acetaldehyde (Km for acetaldehyde as substrate 1.7 mM) and it has two important ROS products, O_2^- and H_2O_2, and the ratio of both products depends on pH and PO_2 (16). It is widely present in mammalian tissues and Table 2 gives data on the organ distribution of xanthine oxidase activity in humans. In 1997 Moriwaki et al. (17) reported sulphite oxidase activity in mitochondria, mainly in the intermembraneous space. This is a member of the same Mo- containing family of enzymes as xanthine oxidase.

Up to now the presence of xanthine oxidase in mitochondria has under debate. In 1982, Mather (18) detected the activity in cow mammary tissue and reported a subcellular distribution of activity as follows: 0% in the nucleus, 5.6% in the mitochondrial fraction, 10.4% in the microsomal fraction and 84% in the postmicrosomal supernatant.

5.2. Importance in physiology and disease

In 2002, Saavedra *et al.* (19) reported that i.v. infusion of allopurinol diminishes mitochondrial O_2 consumption and increases the mechanical efficiency of dog hearts. In 2001, Ukai and coworkers (20) also showed that allopurinol increases the contractile capacity of dobutamine in dogs after physical exercise. These results and other reports (21), seem to indicate a correlation between xanthine oxidase activity and induction of apoptosis on the one hand and on mitocondrial respiration and ATP production on the other.

The results presented here indicate that there is an increased expression of xanthine oxidase in mammary gland mitochondria isolated 12-24 hours after weaning, and an increase in mitochondrial xanthine oxidase activity 24 hours after weaning.

In 2005, Sanganahalli (22) showed in an experimental model in rat brain, that allopurinol, can inhibit the activity of cytosolic xanthine oxidase, reducing the production of ROS and inhibiting mitochondrial depolarization. Our results coincide with those of Sanganahalli, and both point out to the importance of xanthine oxidase in functional mitochondrial changes. In 2004 in an extensive review, Berry and Hare (23) emphasized the importance of xanthine oxidase in the molecular mechanisms and patho-physiology of cardiovascular diseases. The enzyme is an important source of RNS at the level of the vascular endothelium. Therefore the increase in expression of nitrate proteins seems to be due to the increase in xanthine oxidase activity after weaning in mitochondria. The significant increase in nitration of low molecular weight mitochondrial proteins

Table 2. Xanthine oxidase content and activity in human organs

Human Tissue	Xanthine oxidase content (ng/mg tissue protein, and other indicated unit)	Xanthine oxidase activity (nmol urate/min.mg tissue protein; and other indicated units)	References
Liver	146		24
Intestine	556	0.1μM of urate/min/mMTrp	24, 25
Lung		< 0.005	26
Bronchial epithelial cells		60	27
Milk		87 ± 4	28
Heart		< less than 2nU/g	29
Serum	< 5 ng/ml		24

24 hours after weaning coincides with the significant increase in xanthine oxidase expression and activity. Further studies should assess the possible role of mitochondrial xanthine oxidase in the generation of radical nitrogen species and their physiological importance during different mitochondrial-dependent situations like aging, physical exercise or apoptosis.

6. PERSPECTIVES

Mitochondria are key organelles in a wide range of cell mechanisms. Here we present evidence indicating that mitochondrial xanthine oxidase activity could play an important role in several physiological and pathological circumstances. Further studies should address the contribution of xanthine oxidase to the mitochondrial production of ROS and NO, considering that nowadays only the electron transfer chain and the mitochondrial nitric oxide synthase are considered as the physiological sources of such compounds.

7. ACKNOWLEDGEMENTS

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