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XANTHINE OXIDASE/DEHYDROGENASE ACTIVITY IN INTACT CULTURED CELLS (*IN SITU* ANALYSIS)

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The measured ratio of xanthine oxidase activity to the total activity of xanthine oxidase and dehydrogenase showed higher values in intact cells than when similar cells were homogenized. The total activity was the same for both systems. The xanthine oxidase ratio was 90, 60, 50, 50, 60% in V79, RIF/Ha[3], SCC7, KHT intact cells and freshly extracted murine peritoneal macrophages respectively while the corresponding ratios measured were 25, 40, 38, 35, 22% when the cells were lysed by homogenization. Superoxide radical $O_2^{\cdot-}$ production by addition of xanthine to intact or homogenized cells to activate intracellular xanthine oxidase was higher in intact than homogenized cells. Homogenization of cells and tissues in the presence of dithioerythritol (DTE) can evidently lead to a considerable under-estimation of the xanthine oxidase ratio. The effect of hypoxia on cells has also been examined.

KEY WORDS: Xanthine oxidase, cultured cells, macrophages, superoxide radicals, NADH, lactate dehydrogenase.

INTRODUCTION

The conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO) in hypoxic and ischaemic tissues[1,2] and the accumulation of hypoxanthine[3] is one of the proposed mechanisms in the pathogenesis of tissue damage due to oxygen toxicity after reperfusion of hypoxic and ischaemic tissues.

Evidence for the pathological role of these processes has been obtained in animal models of ischaemic damage to the intestine, heart, lung and brain.[1,3,4] Localization of xanthine oxidase activity has been detected by different techniques in microvascular endothelial cells[5] and endothelial cell damage has been observed at an early stage of pulmonary oxygen toxicity[6] and in ischaemic reperfusion injury.[7] There have been no *in vivo* or *in situ* assays carried out for the separate estimation of XO and XD activity and neither the histochemical nor the immunological assays have distinguished between XO and XD. Assay of XO and XD is usually carried out on homogenized tissues or cells. We have attempted to assay for both XO and XD activity and superoxide radical production by adding xanthine to activate intracellular xanthine oxidase in both intact and homogenized cells.

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MATERIALS AND METHODS

Cell Culture

Chinese hamster V79 cells and mice tumour cell lines SCC7, KHT and RIF were grown at 37°C in 95% air + 5% CO_2 in monolayer flasks in Eagle's Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS).

Macrophages

Macrophages were extracted from 6 month-old CBA male mice obtained from the MRC Radiobiology Unit animal house. The mice were killed by cervical dislocation, the abdominal wall was exposed by cutting away a flap of skin and 5 ml of cold PBS (phosphate buffered saline) injected in the abdominal cavity. The PBS-filled abdomen was gently massaged to mix in the macrophages. The macrophages, extracted by drawing out the PBS suspension, were centrifuged, resuspended in PBS and immediately assayed for XO/XD activity.

Cell Counting

0.5 ml of trypan blue (0.4%) solution was added to 0.5 ml of cell suspension and mixed thoroughly. After 5–15 min cells were counted using a haemocytometer.

Hypoxia

Cells were made hypoxic by passing 95% N_2, 5% CO_2 over the surface of cell suspension at a flow rate of 500 ml/min at 37°C.

Cell Preparation

The culture medium was discarded and the cells washed with PBS before trypsinization of the cells for 2 min. The action of trypsin was terminated by addition of culture medium containing FCS. The cells (*ca.* 2×10^7) were centrifuged and resuspended in either PBS (2 ml) to assay for enzyme in intact cells or in 50 mM phosphate buffer, pH 7.4, containing 10 mM dithioerythritol (DTE), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM EDTA to assay for enzyme in homogenized cells. DTE and PMSF were added to stop (as it is usually assumed) any artificial conversion of XD to XO during homogenization. These cells were homogenized using Ultra-Turrax T25 homogenizer and centrifuged (100,000 *g*) for 20 minutes and the supernatant used for enzyme assay.

Enzyme Assay

The activities of xanthine oxidase (XO) and xanthine dehydrogenase (XD) were assayed at 37°C by a fluorimetric technique[8] using a SLM-8000 fluorimeter set to 345 nm excitation and 390 nm emission. In this method, a measurement is made of the emission from cell homogenates or suspensions of intact cells in cuvettes. Activity of XO was determined by measurement of the rate of oxidation of pterin (2-amino-4-hydroxypteridine) as indicated by the linear increase in emission following addition of 20 μl of fresh 1 mM pterin solution. The combined activity of XO and XD was

TABLE I

XO ratio (%) in intact and homogenized cells. Values are shown as the average of experiments carried out and were within 95% range. Total activity is expressed as μM of product (isoxanthopterin)/10^6 cells/10 min at 37°C

Cell line	Intact	Homogenized	Number of experiments	Total activity
CH-V79	90	25	> 10	0.05*
RIF/Ha[3]	60	40	2	0.065
SCC7	50	38	3	0.075
KHT	50	35	2	0.045
Macrophages	60	22	4	0.04**

*Average of total activity in intact cells is 0.0496 versus 0.0492 in homogenized cells.
**Average of total activity in intact cells is 0.0385 versus 0.0408 in homogenized cells.

finally determined from the increase in the intensity of emission that follows addition of 20 μl of 1 mM solution of methylene blue.[8] The reaction was inhibited by addition of 20 μl of 1 mM allopurinol. The activity of XO is expressed as a percentage of the total enzyme activity. Isoxanthopterin was added after the inhibition of the reaction in appropriate concentrations to serve as internal standard. The validity of the assay procedure has been demonstrated by the consistency achieved with large numbers of different samples using both the fluorimetric assay mentioned and comparison with the traditional spectrophotometric assay by following the amount of uric acid formed from xanthine without NAD^+ (XO activity) and in the presence of NAD^+ (XO + XD activity).[5,8,9]

The production of NADH was followed by the addition of xanthine (50 μM) and NAD^+ (500 μM) by exitation at 340 nm and emission at 450 nm.

Superoxide Radical (O_2^- assay)

Intact cells 1 mM Diethyldithiocarbamate (DDC), a superoxide dismutase (SOD) inhibitor, was exposed to a monolayer of cells for 1 hr. The culture medium and DDC were washed off and 3 ml of PBS containing 50 μM xanthine and 10 μM cytochrome C were added. The cells were incubated at 37°C for 15 min. Allopurinol (1 mM) was added to stop the reaction. The generation of O_2^- was detected by measuring the reduction of cytochrome C at 550 nm in cell medium.[9]

Homogenized cells Cultured cells were homogenized in PBS containing 10 mM DTE, 1 mM PMSF, 0.5 mM DDC. The homogenates were centrifuged and the supernatant was taken to assay for O_2^- production by adding 50 μM xanthine and 10 μM cytochrome C.

Materials

All chemicals used in this study were obtained from Sigma Chemical Company.

RESULTS AND DISCUSSION

The ratios of XO activity to the combined activity of XO and XD determined using the procedure described in intact cells are higher than for the supernatant of homogenized cells as shown in Table I. The combined activities of XO and XD measured

when the assay was carried out with samples from both intact or homogenized cells were the same within the limits of experimental error. Allopurinol inhibited both oxidase and dehydrogenase reactions in intact cells as well as in homogenates.

The system listed in Table I were investigated further by measuring the production of NADH due to the reaction of XD with NAD^+ in presence of pterin or xanthine as substrates for XO/XD. It was found that more NADH was produced in homogenized than in intact V79 cells. This is consistent with the higher proportion XD activity in homogenized than in intact V79 cells.

To ascertain whether there is locally enough NAD^+ within intact cells to completely trigger the combined XO + XD reaction when pterin or xanthine are added, any NAD^+ present in intact V79 cells was converted enzymically to NADH by addition of 20 μl of lactic acid at different concentrations (1–5 mM solution). It was found that neither XO nor XD activity was affected by addition of lactic acid. Direct measurement showed that no NADH was produced in intact V79 cells upon addition of xanthine or pterin without the addition of NAD^+ to the system.

This result was checked by showing that endogenous lactate dehydrogenase activity can be measured in intact V79 cells by following the production of NADH which occurs upon the addition of lactic acid (10 μM) and NAD^+ (10 μM) to the cell suspension. This confirms that the addition of lactic acid would have brought about conversion of NAD^+ to NADH. Although it may be difficult for NAD^+ to enter intact cells it has been reported that addition of exogenous NAD^+ to NAD^+-deprived cells in culture has restored intracellular NAD(H) within 4 hr.[20] This requires further investigation, but our results showed that addition of xanthine to intact cells followed by NAD^+ results in the immediate formation of NADH, indicating an intracellular interaction between NAD^+ and XD. It is widely accepted that xanthine oxidase/dehydrogenase is localized in cell cytoplasm[5,7] and so it appears that NAD^+ has penetrated the cell membrane by some mechanism. Xanthine oxidase in the cell membrane could achieve the observed result.

Addition of DTE (10 mM) and PMSF (a protease inhibitor) (1 mM) to intact V79 cells did not change the measured XO% but incubation of V79 cells with DTE (10 mM) but without PMSF for 15 min at 37°C lowered XO ratio from 90% to 70%.

Similar results showing enhanced XO levels in intact cells were found with the various tumour cell lines although the determined oxidase levels are not as high as in the V79 cells. Evidently the effect we observe is a general one not restricted to V79 cells.

The measurements of XO/(XD + XO) ratios in freshly isolated murine peritoneal macrophages are particularly interesting since in this case the cells probably represent most closely the true situation in normal mammalian cells (*in situ*). It was found that the proportion of XO in macrophages depended on the extraction and assay procedure followed as shown by data given in Table II. If the XO% was measured in intact macrophages extracted in PBS only, the ratio was 60% in contrast with 22% when the cells were homogenized in presence of DTE and PMSF. This is consistent with the behaviour and comparable with values in other cell lines tested. Addition of DTE has been reported by Panus *et al.*[17] to convert a reversible form of XO to XD.[11] This could explain why XO% ratios are lowered from 60% in intact cells isolated in PBS only to values between 22 and 28% when DTE is added to the intact cells during and after extraction. Table II also shows that the presence of DTE in the homogenization buffer results in lower XO ratios. Homogenization of V79 cells in buffer containing PMSF only without DTE resulted in high XO ratios ($\sim$ 70%) in contrast to 25%

TABLE II
XO ratio in murine peritoneal macrophages. Values are the average of 4 experiments ± SD

Extraction Buffer	XO% Intact	XO% Homogenized
PBS	60 ± 2.89	22 ± 2.25 (DTE: 10 mM, PMSF: 0.5 mM) added during homogenization
PBS	60 ± 2.89	40 ± 3.12 (no DTE or PMSF)
PBS containing DTE, PMSF (10 mM, 0.5 mM)	22 ± 1.85	22 ± 2.15
PBS	28 ± 3.45 (DTE, PMSF added after extraction)	22 ± 2.25 (DTE, PMSF added during homogenization)

in presence of DTE and PMSF. Adding DTE only without PMSF resulted in low XO ratios. KHT cells also behaved similarly. Evidently high XO ratios in absence of PMSF and DTE are not due to proteolysis of the enzyme which occurs during homogenization. These results show that the measured XO/XD ratio changes due to effects arising from treatment of cells and homogenates.

The measurement of O_2^- production in intact and homogenized V79 cells provides an alternative procedure for estimating the ratio of XO in total enzyme. It was not possible in the first attempts to assay for O_2^- production in intact V79 cells. Precipitation and heterogeneity of the cell suspensions resulted in erratic behaviour of the absorption of cytochrome C reduction at 550 nm so we finally used monolayer cell culture. Even in monolayers there was no detectable cytochrome C reduction upon the addition of xanthine and cytochrome C without addition of DDC, which serves as a superoxide dismutase inhibitor. It is reported[12] that V79 cell extracts can be used to inhibit the reduction of cytochrome C because of the presence of SOD so we included DDC in all our O_2^- assays. Table III shows that the increase in absorption at 550 nm is significant in intact cells and greater than the increase in absorption in homogenized cells, consistent with higher XO activity in intact cells. Addition of SOD and/or allopurinol inhibited the reduction of cytochrome C, so we conclude that the reduction of cytochrome C was due to xanthine oxidase O_2^- radicals.

The results reported in Table III shows the intact V79 cells generate 4 pmoles of O_2^- from 10^6 cells/min which is consistent with the values previously determined for endothelial cells.[9,13]

The *in situ* enzyme activity measurements show that in all cases for intact cells the xanthine oxidase enzyme behaves mainly as the oxidase, dehydrogenase activity being least in the cell systems examined. This disagrees with the conclusion of Waud and Rajagopolan[14] (p. 378) based in assays on homogenized cells in the presence of inhibitors. Our results clearly demonstrate that homogenized cells do not yield the true state of oxidase activity in intact cells.

In homogenized normal tissue reported XO% values tend to be between 15–30%[15,16] and as high as 70–100% in tumours[17] and ischaemic tissues.[16] Assays using homogenized material for the % of oxidase which we have made in a wide variety of different animal tissues including lung, brain, and liver as well as cultured cells

TABLE III

Superoxide radical ($O_2^{\cdot-}$) production as measured by the increase of absorption at 550 nm due to cytochrome C reduction. In the control, allopurinol was added at the start of the reaction

(a) aerobic V79 cells (average of 6 independent experiments ± SD)

	Intact V79 cells	Homogenized cells
Control:	0.2580 ± 0.001	0.3087 ± 0.007
Experiment:	0.2835 ± 0.018	0.3137 ± 0.009
ΔA 550 nm:	0.0255	0.005
	$P < 0.001$*	$P < 0.1$**

(b) hypoxic V79 cells (average of 4 independent experiments ± SD)

	Intact	Homogenized
0 hr:	0.3585 ± 0.11	0.3220 ± 0.006
16 hr:	0.3608 ± 0.014	0.3310 ± 0.006
ΔA:	0.0023	0.01
	$P < 0.1$**	$P < 0.02$*

Calculated $O_2^{\cdot-}$ production for V79 cells:
4 pmole/10^6/min
Significance of changes in ΔA 550 nm is calculated as P values using t-test for the comparison between the control and the experiment.
*Significant.
**Non-significant.

(unpublished results) are consistent with these published values and we can see no reason why the measurements on the states of the enzyme in intact cells using essentially the same technique should not be valid. The reactions involved in the assay upon addition of substrates and inhibitors were the same in both intact cells and the homogenized extracts. The reliability of the fluorimetric assay method is strongly supported by the finding that in all the systems the *total* enzyme activity for both oxidase and dehydrogenase was the same in intact and homogenized cells.

The results obtained can be interpreted within the framework of previous investigations[14,16] on interchange between oxidase and dehydrogenase states of the enzyme. The labile form of the dehydrogenase enzyme which is assayed after homogenization is transformed into the oxidase form by proteolysis, heating, organic solvent etc. On the basis of observations on a variety of such effects it has been postulated that an intermediate exhibiting *both* oxidase and dehydrogenase activity exists of reversible interconversion between two enzyme states which have unique dehydrogenase or oxidase activity[18,19]. All of these states can form a non-reversible form of the enzyme with oxidase activity, presumably by proteolysis. The reversible forms of the enzyme have molecular masses of 150,000 daltons in contrast to 130,000 for the irreversible oxidase form.[14,16]

The data reported in this paper are consistent with a situation where in the intact cell the enzyme exists mainly in the oxidase (reversible and irreversible) form which on homogenization is converted predominantly into the XD form due to the presence of DTE in the homogenization medium. This supposition is confirmed by the observation that in intact macrophages DTE converts all the reversible enzyme into XD when added before or after extraction.

In intact V79 cells there is little difference between aerobic and hypoxic cells which

have similar XO ratios *in situ*. In contrast for homogenized cells there is a greater difference between hypoxic and aerobic cells indicating higher XO ratio in hypoxic homogenates (Hasan *et al.*).[10] It follows from these results that the effect of hypoxia or ischaemia on cells is to bring about the conversion of XD to irreversible XO so the homogenization of cells in presence of DTE cannot reconvert XO to XD resulting in a high value for XO in hypoxic homogenates. We have also shown (Hasan *et al.*)[10] that changes in lipid peroxidation markers occured earlier than conversion of XD to XO upon exposure of V79 cells to hypoxia. Also there was no burst or increase in investigated lipid peroxidation markers upon reoxygenation of cells. This supports our finding that in intact cells the XO ratio is high although it could be mainly in a reversible form in the aerobic state and an irreversible form in the hypoxic state.

In summary it must be concluded that in intact cells the xanthine oxidase enzyme has higher XO activity than in the homogenized condition. In the intact cell the enzyme must exist in a state exhibiting oxidase activity which is converted during homogenization in the presence of dithioerythritol (DTE) to the XD form. This has considerable implications for understanding the mechanism underlying oxidative effects which occur in biological systems exposed to ischaemia followed by reperfusion.

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