Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 886-891

OXYGEN ENHANCES IN VIVO MYOCARDIAL SYNTHESIS OF POLY(ADP-RIBOSE)*

Q. P. GHANI AND M. HOLLENBERG

Cardiovascular Research Institute and
Departments of Medicine
Veterans Administration Hospital and
University of California
San Francisco, California 94121

Received February 14, 1978

Summary:

In vivo synthesis of poly(ADP-ribose) is demonstrated in cultured chick embryo heart cells. Cells grown with (^{14}C)ribose incorporate 28 - 31% more radioactivity into poly(ADP-ribose) in 20% O_2 (in which they divide more slowly) than in 5% O_2. Reaction product was identified as poly(ADP-ribose) by its insensitivity to various enzymes and by its digestion with snake venom phosphodiesterase to phosphoribosyl-AMP and AMP. Poly(ADP-ribose) glycohydrolase activity was similar in 20% and 5% O_2. Thus, both poly(ADP-ribose) polymerase activity (shown in an earlier study) and poly(ADP-ribose) increase in cells growing more slowly in 20% vs 5% O_2. These data suggest that poly(ADP-ribose) metabolism participates in the regulation of heart cell division by O_2.

Introduction:

Various workers have suggested that poly(ADP-ribose) may be involved in the regulation of cell growth and differentiation (1-3). In previous studies we have shown that cultured chick heart cells divide more rapidly in 5% oxygen than in 20% oxygen (4) and that the activity of the enzyme poly(ADP-ribose) polymerase varies inversely with the rate of cell division (5). In the more slowly dividing cells grown in 20% oxygen the greater polymerase activity observed (as measured in an in vitro assay) was assumed to result in greater rates of synthesis of product in vivo, i.e., of poly(ADP-ribose). The present studies confirm the in vivo synthesis of poly(ADP-ribose) in chick embryo heart cells grown in tissue culture. Furthermore, they compare the rates of incorporation in vivo of (^{14}C)ribose into the newly synthesized poly(ADP-ribose) of heart cells grown in 5% and 20% oxygen.

* This investigation was supported by U.S. Public Health Service Grant Program Project HL 06285 and the Medical Research Service of the Veterans Administration.

0006-291X/78/0813-0886$01.00/0

Materials and Methods:

AMP, ATP, ADP-ribose, adenosine, NAD^+, and pronase were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ribonuclease, deoxyribonuclease and snake venom phosphodiesterase were bought from Worthington Chemical Co., Freehold, New Jersey, USA. D-(1-^{14}C)ribose was purchased from Amersham/Searle, Chicago, Il, USA.

Heart cells from 8 day chick embryo were grown in 5% and 20% oxygen as described previously (4). After three days of growth 20 µCi of (^{14}C)ribose (50mCi/mmol) were added to each plate (per 3 ml of media) and cells were allowed to grow for an additional 24 hours. Cells were washed with 0.1 M Tris-HCl pH 7.4, harvested and treated with ice cold 10% TCA for 30 minutes. The precipitate was collected by centrifugation (15,000 x g for 30 min), washed with cold 5% TCA and with 95% ethanol to remove free radioactivity, and then suspended in 2 ml of 0.1 N NaOH and incubated at 37°C for 60 min. After neutralization with concentrated HCl, Tris/HCl (pH 7.5), $MgCl_2$, and $CaCl_2$ solutions were added to final concentrations of 50 mM, 10 mM and 1 mM respectively. To eliminate the contaminating nucleic acids, DNAase and pancreatic RNAase were added to a final concentration of 100 µg/ml and the mixture was incubated for 60 min at 37°C. After centrifugation (15,000 x g for 15 min) the supernatant was deproteinized by extraction with chloroform/3 methylbutane-1-ol (24:1 v/v). 0.1 vol of 25% (w/v) potassium acetate, pH 5.0, was added to the aqueous layer and the polymer was precipitated by the addition of 3 vol of cold 95% (v/v) ethanol. After 24 h at -20°C the mixture was centrifuged (15,000 x g for 15 min), the precipitate dissolved in 10 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ and counted in a liquid scintillation counter. To determine the chemical characteristics of the isolated ^{14}C-labeled TCA precipitable compound it was subjected to digestion by DNAase, RNAase, trypsin, NADase, and snake-venom phosphodiesterase. The digestion products were characterized by paper chromatography in the following solvent system: isobutyric acid/n-propanol/water/NH3 (75:25:25:2.5 ml of 25% solution). To evaluate the rate of poly(ADP-ribose) degradation in the two different oxygen concentrations poly(ADP-ribose) glycohydrolase was assayed according to the method by Burzio et al. (6)

Results:

At 96 hours of growth and after incubation in (^{14}C)ribose for 24 hours, heart cells grown in 20% oxygen contain 28 - 31% greater net radioactivity in poly(ADP-ribose) than do cells grown in 5% oxygen (Table 1). Incorporation of (^{14}C)ribose into poly(ADP-ribose) was measured by determining the activity of the final TCA insoluble product. Although (^{14}C)ribose is also incorporated into DNA, RNA, and lipids these substances are eliminated by the isolation steps. Thus, as shown in Table 2, the product is insensitive to DNAase, RNAase, trypsin and NADase but is digested almost completely (>98%) by snake venom phosphodiesterase. When the digestion products were separated by paper chromatography most of the applied radioactivity could be accounted for by two spots, one migrating with 5'AMP and the second at the position of phospho-

Table 1

(^{14}C)ribose incorporation into poly(ADP-ribose)

	Counts per minute per mg protein		% increase
	5% O_2	20% O_2	
Expt. 1 (8 plates)	1178	1540	30.7
Expt. 2 (6 plates)	990	1273	28.6

Plates were pulsed with 20 μCi (^{14}C)ribose (50 mCi/mmol) at 72 hours of growth and cultures allowed to grow for an additional 24 hours. Poly(ADP-ribose) was isolated as described under Methods in text.

ribosyl-AMP (Figure 1). Thus, essentially all of the TCA insoluble material is poly(ADP-ribose). The rate of degradation of poly(ADP-ribose) glycohydrolase is similar in homogenates of cells cultured in 5% and 20% oxygen (Table 3).

Discussion:

In an earlier study (5) we showed that heart cells grown in 20% oxygen divide more slowly and exhibit greater poly(ADP-ribose) polymerase activity than those in 5% oxygen. The present studies extend these findings and demonstrate the presence, in vivo, of poly(ADP-ribose) in cultured heart cells. The natural presence of poly(ADP-ribose) in various other types of cultured cells has been demonstrated by Kidwell and associates (7,8) who quantified its amount by pulse labeling with (2-^{3}H)adenosine and by radioimmunoassay. In HeLa cells both techniques provided similar results. In the present studies (^{14}C)ribose was used to label the precursor NAD^+ which was then incorporated into poly(ADP-ribose). Such pulse label techniques showed that heart cells grown in 20% oxygen contain more poly(ADP-ribose) than do those in 5% oxygen. The radioactivity measured in poly(ADP-ribose) represents a balance between synthesis and degradation. The greater radioactivity in poly(ADP-ribose) from cells grown in 20% oxygen reflects mainly greater net synthesis of poly(ADP-ribose) in vivo since the activity of the main degrading emzyme, poly(ADP-ribose)

Table 2

Enzyme digestion of final TCA insoluble product

Enzymes (μg/ml)	Remaining acid insoluble radioactivity (c.p.m.)			
	Expt. 1		Expt. 2	
	5% O_2	20% O_2	5% O_2	20% O_2
None	553	750	422	630
Ribonuclease (100) plus deoxyribonuclease (100)	541	739	443	618
Trypsin (100)	521	721	450	607
NAD glycohydrolase (100)	520	715	438	638
Snake-venom phosphodiesterase (40)	33	46	23	36

Isolation of the final TCA precipitate is described under Methods in the text. This was incubated with the indicated enzymes in appropriate buffers (5) for 60 min at 37°C. The reaction was stopped by adding cold 10% TCA, the acid-insoluble material collected on a Millipore filter, washed with cold 5% TCA and the remaining radioactivity determined by liquid-scintillation counting.

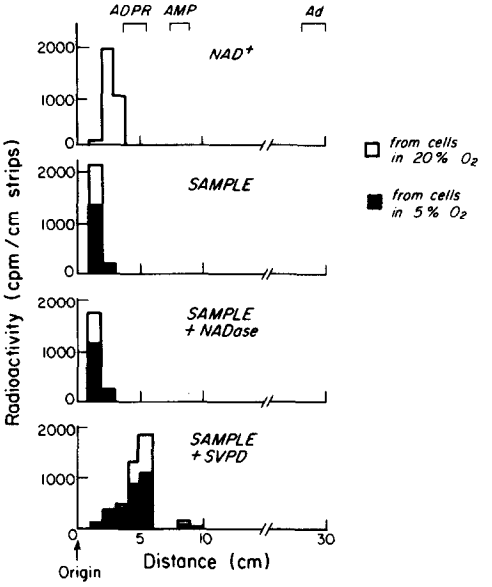

Fig. 1 Identification of poly(ADP-ribose). Note near complete digestion of sample with snake venom phosphodiesterase. ADP-ribose which migrates similarly to phosphoribosyl-AMP was used as a marker since the latter is unavailable commercially.

Ad = Adenosine
ADPR = ADP-ribose
SVPD = Snake venom phosphodiesterase

Table 3

Poly(ADP-ribose) glycohydrolase activity in cardiac cells grown in 5% and 20% oxygen

Source of glycohydrolase	Activity Remaining (c.p.m.)	Polymer degraded (c.p.m/μg protein)
Control (0 enzyme)	12,509	-
Cell homogenate, 5% O_2 (10 μg protein)	10,696	181
Cell homogenate, 5% O_2 (50 μg protein)	4,351	163
Cell homogenate, 20% O_2 (10 μg protein)	10,851	166
Cell homogenate, 20% O_2 (50 μg protein)	4,229	166

The reaction mixture contained 12,500 cpm of poly((^{14}C)ADP-ribose) prepared and isolated by the method of Burzio et al (6) using poly(ADP-ribose) polymerase from rat liver nuclei, 50 mM potassium phosphate buffer pH 7.0, 5 mM 2-mercapto ethanol, 0.5 mM NaF to inhibit phosphodiesterase activity and 10 - 50 μg of protein from cell homogenates in a total volume of 0.3 ml. The reaction mixture was incubated for 10 min at 37°C and the remaining polymer was precipitated by the addition of 3 ml of 10% cold TCA. The precipitate was collected on a millipore filter, which after washing was dissolved in 15 ml Bray's solution and the radioactivity measured in a scintillation counter. Glycohydrolase activity is represented by the difference between the amount of radioactivity left on the filter in the control sample compared with that of the sample containing enzyme and is expressed as polymer degraded (c.p.m./μg protein).

glycohydrolase, was similar in both oxygen concentrations. We have yet to measure pool size, either of ribose or NAD^+, without which one cannot rigorously equate (^{14}C)ribose incorporation into poly(ADP-ribose) with net synthesis. However, if the pool size of the immediate precursor, NAD^+, did change with oxygen concentration it would probably increase in the cells exposed to higher oxygen for reasons cited below. Specific activity of NAD^+ would decrease as would radioactive incorporation into product rather than increase as occurred in this study. In density inhibited, non-dividing cells or in slowly dividing cells (such as occurs in the higher oxygen concentration) NAD^+ levels appear to be significantly higher than in rapidly dividing cells (9,10). Thus, one would anticipate higher NAD^+ levels in 20% (in which cells grow more slowly) than in

5% oxygen. Additionally, at higher oxygen concentrations NADH would shift to NAD^+ and thus further augment the pool of this immediate precursor of poly(ADP-ribose). Thus, it seems likely that the increased radioactivity in poly(ADP-ribose) does reflect increased synthesis of this substance in 20% versus 5% oxygen. These data, together with those from a previous study which showed that poly(ADP-ribose) polymerase activity increased in cells growing more slowly in 20% than in 5% oxygen (5), suggest that poly(ADP-ribose) metabolism participates in the regulation of heart cell division by oxygen.

References:

1. Hilz, H. and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76,1-58.

2. Claycomb, W.C. (1976) Biochem. J. 154,387-393.

3. Roberts, J.H., Stark, P., Giri, C.P. and Smulson, M. (1975) Arch. Bioch. Biophys. 171,305-315.

4. Hollenberg, M. (1971) Circ. Res. 28,148-157.

5. Ghani, Q.P. and Hollenberg, M. (1978) Biochem. J. 170,387-394.

6. Burzio, L., Riquelme, P.T. and Koide, S.S. (1975) Analy. Biochem. 66,434-445.

7. Kidwell, W.R. and Burdette, K.E. (1974) Biochem. Biophys. Res. Comm. 61,766-773.

8. Kidwell, W.R. and Mage, M.G. (1976) Biochem. 15,1213-1217.

9. Jacobson, E.L. and Jacobson, M.K. (1976) Arch. Biochem. Biophys. 175,627-634.

10. Morton, R.K. (1958) Nature (London) 181,540-542.