Vol. 108, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1982 Pages 174-181

RESTORATIVE EFFECTS OF SPERMINE ON OXIDATIVE PHOSPHORYLATION AND RESPIRATION IN HEAT-AGED MITOCHONDRIA

J.E. Phillips and R.R.J. Chaffee

Department of Ergonomics
University of California
Santa Barbara, California 93106

Received July 27, 1982

SUMMARY: Samples of fresh rat liver mitochondria were heat-aged in 37 °C isotonic sucrose sufficiently to cause approximately a 30% reduction in the ADP:O ratio when using beta-hydroxybutyric acid as substrate. Subsequent polarographic assays showed spermine concentrations between 0.0157 and 0.250 mM to cause a striking linear restoration of the ADP:O ratio. Spermine also improves both the respiratory rate during the conversion of ADP to ATP and the respiratory control ratio of heat-aged mitochondria. The effects of spermine on the respiratory rate after ADP to ATP conversion vary depending on the spermine concentration. Spermine has no significant effects on mitochondrial respiration prior to addition of ADP.

INTRODUCTION

In the past many investigators have postulated that polyamines can stabilize, protect and preserve various bacteria and other cells against deleterious agents (see 1-3). Others have described the stabilization of mitochondrial membranes by certain polyamines (4,5). Some investigators have also presented evidence that polyamines induce, and/or change the rate of mitochondrial volume oscillations (6). All these studies are interesting in that they indicate that polyamines play a physiological role in bacteria and their homologues, the mitochondria. However, there were no detailed publications in the literature specifically designed to investigate effects of polyamines on mitochondrial oxidative and phosphorylative processes previous to studies in this laboratory (7-10). These studies were of particular significance because they were performed on rat liver mitochondria, at a reaction mixture concentration of Mg^{++} within the range of the calculated free

0006-291X/82/170174-08$01.00/0

Mg^{++} levels in the cytoplasm of the rat liver cell (11,12). This obviated the masking effects which occur when one uses higher unphysiological levels of Mg^{++} in the mitochondrial reaction mixture.

In the research for this study we have heat-aged rat liver mitochondria in isotonic sucrose at 37°C for sufficient time to bring about significant reductions in their oxidative and phosphorylative capacities. Samples of these heat-aged mitochondria were subsequently used for polarographic studies on the effects of spermine on these capacities. We have designed our research to determine whether polyamines might have, in addition to protective effects, restorative effects on oxidative and phosphorylative capacities in such heat-aged mitochondria.

METHODS

Mitochondria were isolated from livers of adult male Sprague-Dawley rats by the method described by Hoch and Lipmann (13). All assays were made at 37°C, using the polarographic apparatus described by Estabrook (14). In the liver *in vivo*, acetoacetate is reduced to BOH (15), but if there is an excess of BOH *in vitro*, the reaction is reversed and the oxidation of NADH thus generated can be followed polarographically. For assaying BOH oxidation, a modification of the reaction mixture of Harris et al. (16) was used. The 3.6 ml reaction mixture contained: 900 µMoles sucrose, 90 µMoles KCl, 18 µMoles KH_2PO_4, 72 µMoles Tris-HCl, 1.08 µMoles ADP, 90 µMoles BOH and the appropriate concentrations of spermine and Mg^{++}.

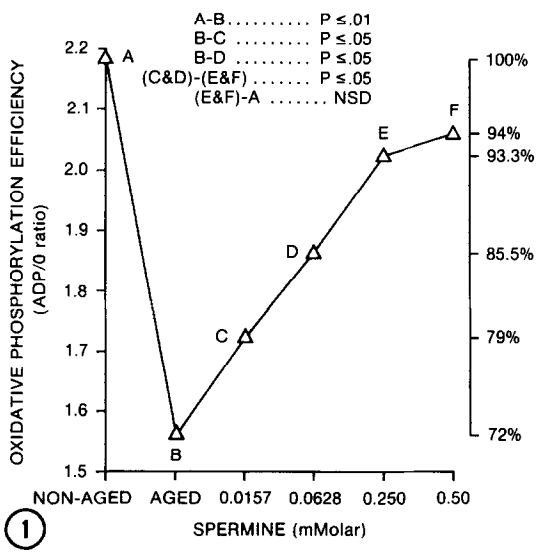
In each assay, 2-4 mg of mitochondrial protein was used. Protein concentration was determined spectrophotometrically by the method of Lowry et al. (17).

Reduction of mitochondrial phosphorylation efficiency and respiration was accomplished *in vitro* by aging the mitochondria at 37°C until approximately 30% of the phosphorylative efficiency was lost. The temperature of the reaction mixture was maintained at 37°C.

Spermine concentration was varied between 0.0157 and 0.50 mM and Mg^{++} concentration was maintained at 0.93 mM, which is within the estimated hepatic levels of unbound cytoplasmic Mg^{++} (11,12). Data were analyzed statistically by correlated T test and further verification was obtained using analysis of variance with the Newman-Keuls Post Hoc Test.

RESULTS

Figure 1 shows the approximate 30% loss in the ADP:O ratio which results from our *in vitro* heat aging regimen. When these heat-aged mitochondria are put into reaction mixtures containing 0.0157, 0.0628 and 0.250 mM spermine there is a linear increase in the ADP:O ratio back to the point where it is not statistically different from control value, i.e. neither E nor F



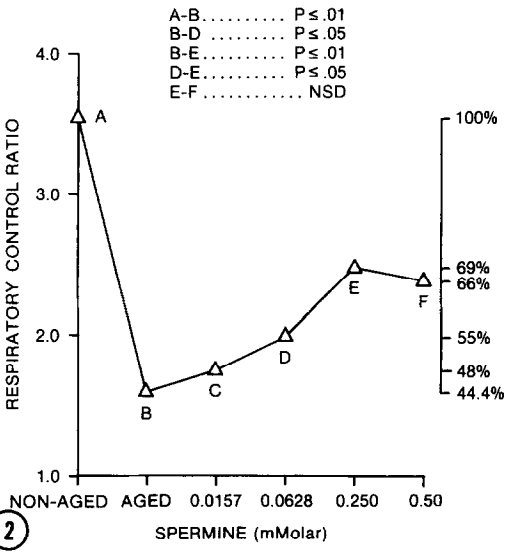


Figure 1: Changes seen in oxidative phosphorylation efficiency due to aging of mitochondria in vitro and after additions of spermine.

Figure 2: Changes seen in Respiratory Control Ratio due to aging of mitochondria in vitro and after additions of spermine.

are statistically different from A. Thus these results indicate that spermine can be a potent polyamine in restoring the oxidative phosphorylative efficiency in heat-aged mitochondria.

As shown in Figure 2, in vitro aging also causes a 55% reduction in the RCR*. Spermine additions result in partial restoration of the lost RCR. At 0.0628 mM there is about a 10% recovery whereas at 0.250 and 0.50 mM there is approximately a 22% to 25% recovery.

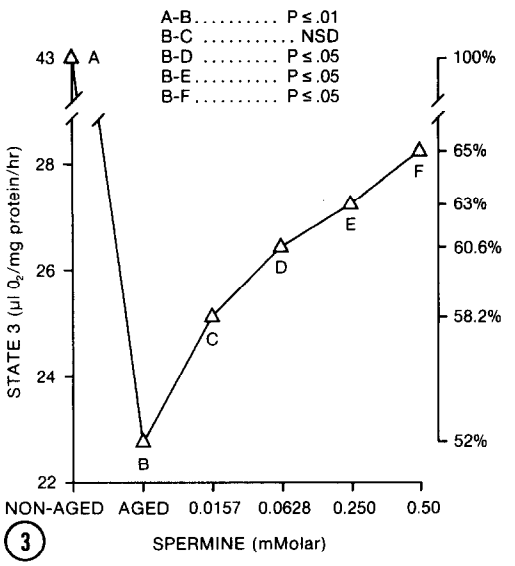
Figure 3 shows that improvements in the RCR are largely due to stimulation in the rate of state 3** respiration. The restorative effects result in an increase in state 3 of aged mitochondria as increasing concentrations of spermine are added. All except the lowest level of spermine (0.0157 mM) produced a statistically significant improvement in state 3 rates when compared to the aged value.

Figure 4 shows spermine to have unusual effects on state 4*** respiration. After in vitro aging the rate of state 4 increases by 12% as compared to

*RCR: Respiratory Control Ratio

**State 3: Respiration during conversion of ADP to ATP

***State 4: Respiration after conversion of ADP to ATP



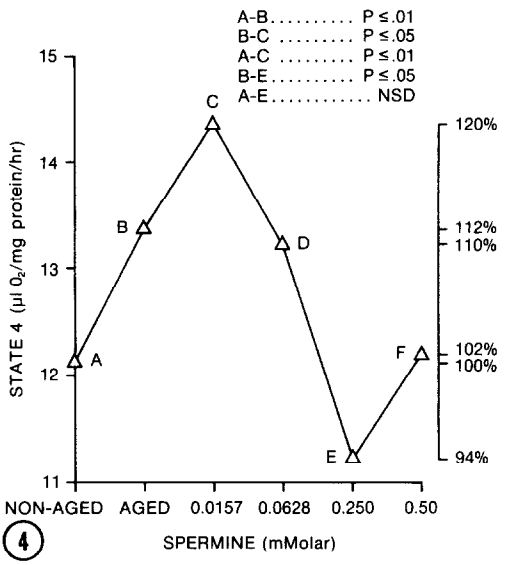


Figure 3: Changes seen in mitochondrial respiration (during ADP-ATP conversion) due to aging of mitochondria in vitro and after additions of spermine.

Figure 4: Changes seen in mitochondrial respiration (after ADP-ATP conversion) due to aging of mitochondria in vitro and after additions of spermine.

control non-aged mitochondria. The presence of 0.0157 mM spermine increases it by an additional 8%, but in the presence of 0.0250 mM or 0.50 mM spermine the aged mitochondria show a restoration of state 4 back to the non-aged (fresh mitochondrial) value.

DISCUSSION

One might at first glance interpret our results as further proof that polyamines in the 37°C reaction mixture are stabilizing the mitochondrial membrane or possibly enhancing mitochondrial longevity, and thus having a generalized protective effect which prevents the continued functional degradation which was initiated in the heat-aging treatment. However, we herein present arguments based on the results of this study which support the concept that spermine has a restorative rather than a stabilizing effect on mitochondrial oxidative and phosphorylative functions.

Our method of heat-aging mitochondria by exposing them to heat at 37°C for 5 or 6 minutes in 8.5% sucrose no doubt causes damage as is indicated by the lowering of state 3 respiration by about 50% and increased state

4 by about 12% (with a consequent lowering of the RCR) and a drop in the ADP:O ratio. Probably some of this damage is permanent, but when the mitochondria are subsequently put into the reaction mixture capable of supporting ATP synthesis, they show indications that the 37°C temperature of the reaction mixture is not in itself causing a continuance of this rapid degradation of mitochondrial function. In fact, the reaction mixture conditions in themselves, sans spermine, apparently cause a stabilization of the membrane with respect to the functions which we assayed polarographically. For example, respiration prior to addition of ADP is linear whether run for one or two minutes in the 37°C mixture of the polarograph, and state 3 respiration (which declines in 5 or 6 minutes in the 37°C sucrose aging treatment) shows no change in slope even if measured for 3 minutes in the reaction mixture. If heat-induced degradation of mitochondrial functions were continuing in the 37°C reaction mixture, one would expect to see, over a 3 minute period, a change in the state 3 respiration slope of about 25%, i.e. a curving downward. Subsequently, there should be a curving upward during state 4 respiration if metabolic control were to continue to decline in the reaction mixture. No such curving occurs: once the mitochondria are placed in the 37°C reaction mixture, respiration prior to the addition of ADP, state 3 and state 4 respiration rates are straight line functions. Thus in the 37°C reaction mixture, in the absence of spermine, there is a relatively stable-state situation which exists throughout the period of polarographic measurements wherein heat exposure at this reaction mixture temperature appears not to be a variable that introduces any serious source of error in the comparisons being made. Thus, with each increment of spermine added to the reaction mixture in successive assays there is a change in the straight line slopes of state 3 and state 4 respiration: neither show any curving with time. This restoration appears to apply with respect to ADP:O ratios also. We interpret this as evidence that: 1. there is a rapid restorative effect which is initiated and completed in a matter of less than a minute (the period in the reaction chamber before measurements commence) during which time the mitochondria come in contact with spermine; and 2. the extent

of these restorative effects appears to be a function of the spermine concentration.

It should be kept in mind that concepts of stabilizing and restorative effects are not mutually exclusive, but the latter would represent a much more significant cellular physiological response. The exquisite sensitivity of the mitochondrial respiratory and phosphorylative performance to a change of as little as 0.0157 mM (15.7 nMoles/ml) in the studies herein presented is much greater than the sensitivity shown with respect to the polyamine-induced stabilization of mitochondria to affect swelling as reported by others (4-6).

There is evidence that rapid shifts in levels of specific polyamines such as spermine in the cell can take place since the enzymes responsible for their synthesis have remarkably short half lives (18-21). This would introduce the possiblity that mitochondrial damage might be counteracted by cellular responses causing changes in cytoplasmic levels of spermine which would improve oxidative phosphorylation efficiency.

Some animal responses have been shown to be associated with changes in cellular polyamine levels, i.e. endocrinological (22-26) and environmental stress induced responses (27). Furthermore, evidence has been presented which indicates that mitochondria isolated from animals acclimated to a stressful environment respond differently to added polyamines than do mitochondria isolated from controls (9). Thus it is not inconceivable that rapid changes in intracellular polyamine levels in cells of animals undergoing physiological changes due to any number of factors may cause restorative effects on mitochondria in vivo.

Unfortunately our experiments do not answer any questions concerning what the specific mechanisms of spermine action are which cause the observed mitochondrial effects on oxidative and phosphorylative functions. However, similar studies we have conducted using putrisine, spermidine, or Mg^{++} as substitutes for spermine show that none of these at concentrations within one order of magnitutde cause the effects that spermine does. Thus the

simplistic idea that spermine acts as a generalized organic bivalent cation or Mg^{++} substitute and thus produces its effects is certainly not tenable. Whatever spermine is doing is most obvious when studies involve the BOH oxidase system, since our results using other substrates have not shown anywhere near such exquisite responses to spermine.

ACKNOWLEDGEMENTS

We wish to thank James R. Allen and Debra K. Mattson for their excellent technical assistance.

We are grateful to the Fund for Integrative Biomedical Research for its support for attendance at the Conference on the Role of Mitochondria in Aging, Washington D.C., Dec. 19, 1980, which enriched our perspective with respect to the research herein presented.

This work was supported by ONR Contract N00014-75-C-0506, NIH Grant A M 18895-01, and NASA-Ames NCA 2-OR-608-601.

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