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Human Aging: Effect on the Activation of Lymphocyte Cyclic AMP-Dependent Protein Kinase by Forskolin[1] (42492)

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Abstract. The effect of forskolin on freshly isolated human lymphocytes was characterized at the level of cyclic AMP-dependent protein kinase. Incubating lymphocytes *in vitro* with this potent adenylate cyclase activator led to time- and dose-dependent activation of protein kinase. The amount of activation varied with the conditions of enzyme preparation, suggesting that lymphocyte cyclic AMP-dependent protein kinase was artifactually activated unless special precautions were taken during its preparation. Under these conditions, forskolin-dependent protein kinase activation was greater in lymphocytes isolated from young compared to elderly human subjects. These results demonstrate coupling between lymphocyte cyclic AMP production and cyclic AMP-dependent protein kinase activation decreases in an age-related manner in man. This decrease resembles the loss of lymphocyte adenylate cyclase activity reported previously and occurs without other changes in lymphocyte protein kinase that were detectable using these methods. The age-related decrease in protein kinase activation is probably accounted for, therefore, by the age-related decrease in the cyclic AMP synthesizing capacity of the lymphocyte.

The cyclic AMP synthesizing capacity of the circulating lymphocyte, among the most accessible human tissues sensitive to hormones and neurotransmitters, decreases during aging (1–5). Similar decreases may account for the refractoriness that develops in the course of human aging to a variety of drugs and hormones which exert their effects through cyclic AMP-mediated pathways in tissues less accessible than the lymphocyte (see Ref. (6) for review).

In the lymphocyte, cyclic AMP is associated principally with growth and differentiation in mammals including man (7, 8). Cyclic AMP synthesis is also stimulated, however, by agonists without growth promoting properties. The importance of cyclic AMP regulation in these cells by β-adrenergic catecholamines and series E prostaglandins remains, therefore, poorly understood although sensitivity to both of these decreases during senesence (9–11).

It is widely acknowledged that cyclic AMP-dependent functions are mediated by cyclic AMP-dependent protein kinase (PK-A) in mammalian cells as initially proposed by Kuo and Greengard (12). In fact, varieties of both PK-A and phosphoproteins specifically associated with growth and differentiation have been described in the human lymphocyte (7, 13). Since PK-A is activated in response to increases in the intracellular concentration of cyclic AMP, changes in its activation could contribute to age-related differences in lymphocyte immune function as well as deterioration of the less well-characterized cyclic AMP-mediated functions regulated by catecholamines and prostaglandins.

In contrast to adenylate cyclase activity which decreases during senescence, we found no age-related changes in the amount of PK-A activity in crude homogenates of circulating lymphocytes isolated from human subjects (14). We now report the effect that subject age has on the activation of lymphocyte PK-A by forskolin, a potent activator of adenylate cyclase in these cells (15, 16). The results indicate the extent to which age-related decreases in cyclic AMP-producing capacity might decrease the cyclic AMP responsiveness of some metabolic pathways in one human tissue.

Materials and Methods. Lymphocytes were isolated from human subjects who were dis-

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ease free and who gave their informed written consent for these studies to be performed. Except for the possible use of alcohol or tobacco, the subjects were unmedicated. The single-step centrifugation method using lymphocyte separation medium (Bionetics Laboratory Products, Kensington, MD) was used as described previously (17). The isolated cells at a density of 2×10^6 cells/ml (determined by hemocytometer counting) were resuspended in freshly prepared Krebs–Ringer bicarbonate buffer without albumin containing 0.15 m*M* 3-isobutyl-1-methylxanthine (IBMX).

In vitro stimulation was carried out by first shaking the intact cell suspensions at 37°C with continual gassing (95% O_2; 5% CO_2) for 30 min. At the end of this pretreatment period, forskolin was added to a final concentration of 70 μM by adding a concentrated solution of the diterpene dissolved in ethanol. An equivalent volume of ethanol alone was added to control cell suspensions which were otherwise handled in an identical manner. Incubation was continued under the pretreatment conditions and after 5 min the cell suspensions were rapidly chilled to 4°C.

The cells were concentrated by centrifugation at 3000*g* for 12 min (at 4°C) and the cell pellet was carefully but quickly rinsed and homogenized using a Teflon–glass homogenizer in ice-cold protein kinase homogenization buffer (0.01 *M* Na^+-phosphate buffer, pH 6.8; 0.001 *M* EDTA) at 4°C at a cell density of 25×10^6 cells/ml. Glycerol was added to the homogenates at a final concentration of 30%. The samples were quickly frozen and stored at −70°C before preparation of the soluble activity as described below. Freezing the homogenate under these conditions preserved the properties of the soluble enzyme activity when it was prepared for up to 2 weeks compared to preparations obtained without prior freezing.

In some experiments an aliquot of each thawed homogenate was withdrawn for determination of total protein kinase activity using the assay described below. Of the remaining homogenate, 0.5 ml was withdrawn and centrifuged at 30,000*g* for 15 min at 4°C to separate the soluble from the particulate subcellular fractions. Dextran-coated charcoal (40 mg) in tablet form (DCC; WestChem Products, San Diego, CA) was routinely added to the homogenate and mixed at 4°C prior to centrifugation. The soluble supernatant fractions were withdrawn for immediate determination of protein kinase activity as follows.

Protein kinase activity was determined by adding 0.02 ml of the cell homogenate or the soluble fraction (the equivalent of 350,000 cells) to 0.13 ml of the assay buffer which was 0.2 *M* Na^+-phosphate (pH 6.5), 0.01 *M* $MgCl_2$, 3.75×10^{-7} mole ATP, and 0.25–0.30 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA), and 50 μg of histone (Type IIAS, Sigma, St. Louis, MO). The assay tubes were incubated at 37°C for 12 min and the reaction was stopped by the addition of 1.5 ml of ice-cold 10% trichloroacetic acid (TCA). The acid insoluble precipitates were collected by filtration onto glass fiber filters and washed four times with 2 ml of ice-cold 5% TCA. The filters were dried and counted in a scintillation cocktail without surfactant.

Tubes containing the complete reaction mixture but without histone were included and processed in an identical manner for subsequent correction by subtraction of histone-independent radiophosphorylation. Using these methods, background phosphorylation in the absence of added histone was <13% of histone phosphorylation. Protein kinase activity was quantified as the nanomoles of radiophosphate transferred to histone per one million cells during the course of the assay.

Results. The protein kinase activity of the human lymphocyte soluble fraction employed in the studies described here was about 75% of the activity of the total cell homogenates described previously (14). The activity of 19 separate soluble preparations, determined in the presence of 2 μM cyclic AMP and using mixed histone as substrate, was 9.1 ± 0.9 nmole/million cells (mean ± SEM). There was no significant difference ($P > 0.05$) between the sensitivity to inactivation by the heat-stable protein kinase inhibitor of the soluble activity used in these studies and that in crude cell homogenates, although the total activity of the soluble fraction was lower than that in the homogenates (data not shown). The specific inhibitor reduced the activity of the charcoal-extracted enzyme from control or forskolin-treated cells 68 ± 5% (mean ± SEM, $n = 8$) when this was determined under the conditions that the activity of crude homogenates

was reduced by 65 ± 3% as reported previously (14).

The regulation of lymphocyte PK-A was quantified by changes that occurred in the protein kinase activity ratio (PKAR): the histone kinase activity in the absence of cyclic AMP in the assay divided by the activity in the presence of cyclic AMP (18, 19). Incubating intact cells with forskolin increased the PKAR from ~0.50 to ~0.75 within 5 min when this was determined using crude lymphocyte homogenates as the enzyme source (Fig. 1). While these results indicate that forskolin exposure activates PK-A in lymphocytes as reported previously (14), the release of cyclic AMP from subcellular compartments upon homogenization frequently leads to artifactual PK-A activation in some kinds of cells (19, 20).

In these instances, charcoal addition adsorbs diffusable cyclic AMP and lowers the PKAR (20). Centrifuging the lymphocyte homogenate in the absence of charcoal addition produced a soluble protein kinase preparation with a PKAR that was significantly greater ($P < 0.01$) than that of the crude homogenate

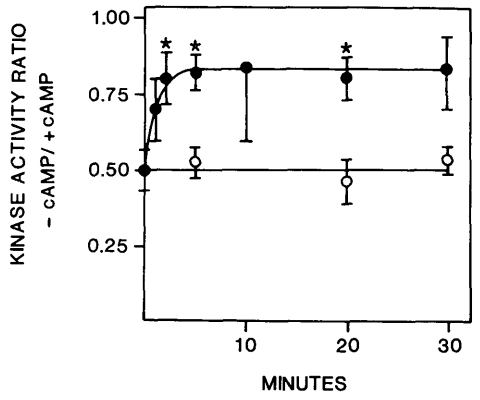

FIG. 1. The effect of incubation *in vitro* with forskolin on human lymphocyte AMP-dependent protein kinase. Freshly isolated human lymphocytes were incubated in the absence (○) or presence (●) of 70 μM forskolin before preparation of the cell homogenate for determination of protein kinase activity in the absence (−cAMP) and in the presence (+cAMP) of cyclic AMP as described under Materials and Methods. The results are the mean ± SEM or four time courses. *Significantly different ($P < 0.05$) from the value obtained at $t = 0$ min in the absence of forskolin when these were compared by the unpaired t test.

TABLE I. THE EFFECT OF CHARCOAL ADDITION ON HUMAN LYMPHOCYTE cAMP-DEPENDENT PROTEIN KINASE ACTIVATION BY FORSKOLIN

	PKAR[b]	
Cellular fraction[a]	Control[c]	+Forskolin[c]
Homogenate	0.48 ± 0.03	0.76 ± 0.02
Soluble	0.65 ± 0.03*	0.82 ± 0.05 (ns)[d]
Soluble + charcoal	0.35 ± 0.03*	0.72 ± 0.08 (ns)[d]

[a] Prepared as described under Materials and Methods.
[b] (activity − cAMP)/(activity + cAMP), the mean ± SEM of results obtained with five to seven separate preparations.
[c] Incubated for 5 min in the absence or presence of forskolin as described under Materials and Methods.
[d] ns, not significantly different from the homogenate.
* Significantly different ($P < 0.01$) from the homogenate by the unpaired t test.

(Table I). In contrast to protein kinase prepared centrifugally from homogenates without the addition of charcoal, the PKAR of the soluble activity was significantly less ($P < 0.01$) than that of the homogenate if charcoal was added before centrifugation (Table II). We investigated the effect that charcoal addition had on the concentration of free cyclic AMP available to activate lymphocyte PK-A in separate experiments using the soluble fraction prepared without prior charcoal extraction. Increasing concentrations of cyclic AMP were added to activate PK-A in the presence of different amounts of charcoal and the aliquots were recentrifuged to remove the charcoal and charcoal-bound cyclic AMP. Charcoal treatment reduced the concentration of exogenously added cyclic AMP ~35-fold as reflected by the magnitude of the rightward shift in the cyclic AMP dose–response curve of lymphocyte PK-A (Fig. 2).

Although charcoal addition lowered the PKAR of cells incubated in the absence of forskolin (the basal PKAR), it did not have a significant effect ($P > 0.05$) on the PKAR of cells treated with forskolin (Table I). Forskolin treatment was associated with an increase in the amount of cyclic AMP-independent activity (determined in the absence of cyclic AMP in the protein kinase assay) and a small decrease in total protein kinase activity (determined by including cyclic AMP in the assay) which was consistant but not significant ($P > 0.05$, Table II).

TABLE II. THE EFFECT OF FORSKOLIN TREATMENT ON LYMPHOCYTE PROTEIN KINASE

Incubation conditions	Protein kinase activity[a] (nmole/million cells) −Cyclic AMP	Protein kinase activity[a] (nmole/million cells) +Cyclic AMP	Forskolin stimulation[b] (-fold)
Control	3.3 ± 0.7	9.7 ± 0.9	—
+Forskolin	5.1 ± 0.4*	9.1 ± 0.9 (ns)[c]	1.9 ± 0.2

[a] Mean ± SEM of seven experiments.
[b] i.e., (PKAR of treated cells/PKAR of untreated cells).
[c] ns, not significantly different ($P > 0.05$) from the activity of the control cells when this was compared by the t test for paired samples.
* Significantly different ($P < 0.01$) than the activity of the control cells when this was compaired by the t test for paired samples.

When determined under these conditions, incubation with forskolin resulted in a dose-dependent increase in the PKAR that characterized activation of PK-A in the charcoal-extracted soluble fraction of the lymphocytes. Using this method, maximum activation occurred when lymphocytes were exposed to forskolin concentrations > 25 μM (Fig. 3). Age had a marked effect on the extent to which exposure to forskolin increased the PKAR, which was significantly lower ($P < 0.025$) in forskolin-treated cells from older compared to younger subjects (Fig. 4). In contrast to the effect subject age had on the PKAR of cells exposed to forskolin, there was no significant ($P > 0.05$) difference in the PKAR of cells from young compared to elderly subjects when lymphocytes were incubated under the same conditions but in the absence of the diterpene.

Discussion. Forskolin treatment activated PK-A in freshly isolated human lymphocytes incubated with the diterpene *in vitro*. Forskolin has been shown previously to be a potent activator of adenylate cyclase in these human

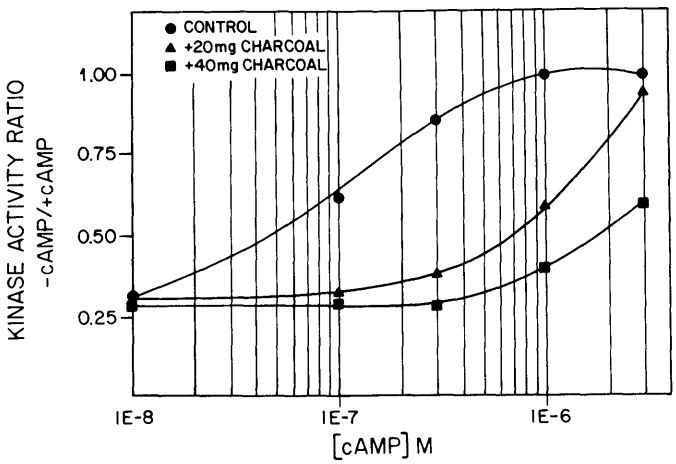

FIG. 2. Activation of lymphocyte protein kinase by cyclic AMP in the absence or presence of charcoal. Soluble protein kinase was prepared by centrifugation of homogenate to which charcoal had first been added as described under Materials and Methods. The amount of charcoal indicated was added to 0.5-ml aliquots of the supernatant fraction which contained the soluble enzyme activity. Cyclic AMP was added to the final concentrations indicated and the mixtures were clarified by recentrifugation. The supernatant was assayed for protein kinase activity in the absence (−cAMP) and in the presence (+cAMP) of cyclic AMP as described. The results are the average of those obtained in two experiments.

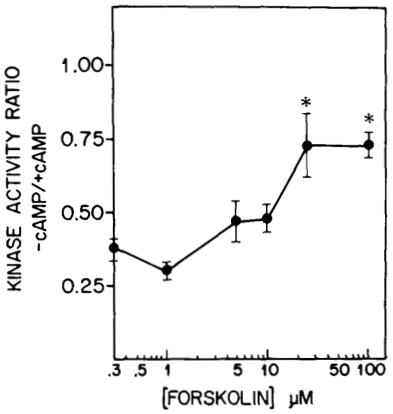

FIG. 3. Forskolin dose-dependent activation of lymphocyte cyclic AMP-dependent protein kinase. The cells were incubated with the indicated concentrations of forskolin before determination of the PKAR of the charcoal extracted soluble fraction as described under Materials and Methods. The values are the mean ± SEM of results obtained with three to seven different preparations. *Significantly different ($P < 0.025$) from the control values, incubated under the same conditions but in the absence of forskolin when the values were compared by the unpaired t test.

cells as it is in mammalian cells in general (15, 16). Activation was reflected by an increase in the lymphocyte PKAR, and the magnitude of the increase was the greatest when it was determined using protein kinase which had been extracted with charcoal. Charcoal addition had its biggest effect on the basal PKAR, where its use resulted in lower values than those of the crude homogenates, which were in turn lower than those of the soluble fraction which had not been exposed to charcoal.

Centrifugation of the homogenate was probably associated with partial PK-A activation unless endogenous cyclic AMP was first removed by charcoal adsorption. This interpretation might explain why the PKAR of soluble PK-A was significantly greater than that of the homogenate unless the centrifugation step was preceeded by charcoal addition, which lowered cyclic AMP levels. Nonspecific cyclic AMP adsorption by the subcellular particulate fraction might account, then, for the lower PKAR of the crude homogenate compared to soluble PK-A prepared without the addition of charcoal. The principal contribution of charcoal addition, therefore, was to increase the magnitude of forskolin activation by lowering the basal PKAR.

Similar artifactual activation of PK-A in the course of its preparation might account for the general absence of cyclic AMP responsiveness attributed to human lymphocyte protein kinase (7, 21–23). Since increased activation of PK-A by forskolin was probably accounted for by increased cyclic AMP production in our studies, artifactual activation of PK-A during its preparation may also account for reports of agonist-dependent increases in cyclic AMP production that occurred in the absence of changes in protein phosphorylation in the human lymphocyte (8).

Aging has been associated with a number of changes in protein kinase activity and many of these changes are tissue as well as species specific (24–26). Age-related decreases of PK-A in its unactivated (holoenzyme) state have been reported to occur in heart, liver, and Leydig cells by some (27–29) but not all (30, 31) investigators. Some of these discrepancies can be accounted for by PK-A changes that are associated with maturation rather than aging, or to changes in the distribution of PK-A between soluble and particulate subcellular fractions (28, 32).

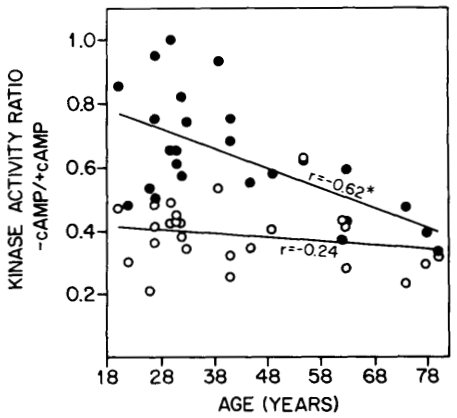

FIG. 4. Effect of subject age on forskolin-dependent activation of lymphocyte cyclic AMP-dependent protein kinase. The PKAR of the charcoal-extracted soluble fraction of lymphocytes from 25 subjects in the indicated age group was determined following incubation in the absence (○) or presence (●) of forskolin as described under Materials and Methods. The values of the correlation coefficients (r) of the lines, drawn using a least squares program, are given. *r value significantly different ($P < 0.01$) from zero.

In our experiments, forskolin exposure was associated with a small but consistent decrease in the total activity of the soluble histone kinase when this was determined by including cyclic AMP in the enzyme assay. This suggests free and activated catalytic subunits of PK-A may be lost to a limited extent from the soluble to the particulate fraction upon homogenization of lymphocytes exposed to forskolin. We determined previously, however, that there was no age-related change in PK-A activity when this was determined using crude lymphocyte homogenates which would have contained PK-A subunits in the particulate as well as the soluble subcellular compartments (14).

Human lymphocyte adenylate cyclase activity, including forskolin-dependent activity, decreases during senescence (1, 2, 16, 33). The age-related decrease in PK-A activation we observed was probably accounted for, therefore, by the deminished cyclic AMP-producing capacity of the lymphocytes from elderly subjects and not by changes in the type or subcellular distribution of protein kinase. Deterioration of the sensitivity of cyclic AMP-dependent pathways that occur in the absence of concomitant changes in PK-A during senescence are not confined to the human lymphocyte (26, 30, 31). Although these may be due to changes in cyclic AMP-sensitive pathways that occur after the PK-A activation step in some cases (26, 31), age-related decreases proximal to this step may best explain the decreases that are encountered in the human lymphocyte.

1. Tam C, Walford R. Alterations in cyclic nucleotides and cyclase-specific activities in T lymphocytes of aging normal humans and patients with Downs Syndrome. J Immunol **125**:1665–1670, 1980.
2. Krall J, Connelly M, Weisbart R, Tuck M. Age-related elevation of plasma catecholamine concentration and reduced responsiveness of lymphocyte adenylate cyclase. J Clin Endocrinol Metab **52**:863–867, 1981.
3. Ebstein R, Oppenheim G, Stessman J. Alzheimer's disease :Isoproterenol and prostaglandin E_1-stimulated cyclic AMP accumulation in lymphocytes. Life Sci **34**:2239–2243, 1984.
4. Lee T-P, Busse W, Reed C. Effect of β-adrenergic agonist, prostaglandins, and cortisol on lymphocyte levels of cyclic adenosine monophosphate and glycogen: Abnormal lymphocyte metabolism in asthma. J Allergy Clin Immunol **59**:408–413, 1977.
5. O'hara N, Daul A, Fesel R, Siekmann U, Brodde O-E. Different mechanisms underlying reduced β_2-adrenoceptor responsiveness in lymphocytes from neonates and old subjects. Mech Ageing Dev **31**:115–122, 1985.
6. Krall J. Aging: Effect on the biochemistry of β-adrenergic catecholamine action. Rev Clin Basic Pharmacol **5**:159–174, 1985.
7. Weber W, Schwoch G, Wielckens K, Gartmann A, Hilz H. cAMP Receptor proteins and protein kinases in human lymphocytes: Fundamental alterations in chronic lymphocytic leukemia cells. Eur J Biochem **120**:585–592, 1981.
8. Parker C. cAMP and lectin-induced mitogenesis in lymphocytes: Possible implications for neoplastic cell growth. In: George WJ, Ignarro LJ, Eds. Advances in Cyclic Nucleotide Research. Raven Press, New York, Vol 9:pp647–660, 1978.
9. Krall J, Connelly-Fittingoff M, Tuck M. Lymphocyte adenylate cyclase and human aging. Proc Soc Exp Biol Med **173**:475–480, 1983.
10. Dillon N, Chung S, Kelly J, O'Malley K. Age and β-adrenoceptor function. Clin Pharmacol Ther **27**:769–772, 1980.
11. Doyle V, O'Malley K, Kelly J. Lymphocyte cyclic AMP production in the elderly: The effect of prostaglandin E_1. Brit J Clin Pharmacol **12**:597–598, 1981.
12. Kuo J, Greengard P. Cyclic nucleotide-dependent protein kinases. IV. Wide-spread occurrence of adenosine 3′, 5′ -monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. Proc Natl Acad Sci USA **64**:1349–1355, 1969.
13. Iyer A, Pishak S, Sniezek M, Mastro A. Visualization of protein kinases in lymphocytes stimulated to proliferate with concanavalin A or inhibited with a phorbal ester. Biochem Biophys Res Commun **121**:392–399, 1984.
14. Krall J, Fernandez E, Connelly-Fittingoff M. Lymphocyte protein kinase activity in cells from young and elderly men and women. Proc Soc Exp Biol Med **180**:462–467, 1986.
15. Stessmann J, Mintzer J, Lipschitz Y, Shemesh Z, Goldin L, Ebstein R. Heritability of forskolin and hormone-stimulated adenylate cyclase activity in human lymphocytes. J Cyclic Nucleotide Protein Phosphorylation Res **10**:317–326, 1985.
16. Abrass I, Scarpace P. Catalytic unit of adenylate cyclase: Reduced activity in aged-human lymphocytes. J Clin Endocrinol Metab **55**:1026–1028, 1982.
17. Krall J, Connelly M, Tuck M. Acute regulation of β-adrenergic catecholamine sensitivity in human lymphocytes. J Pharmacol Exp Ther **214**:554–560, 1980.
18. Soderling T, Corbin J, Park C. Regulation of adenosine 3′, 5′-monophosphate-dependent protein kinase. II. Hormonal regulation of the adipose tissue enzyme. J Biol Chem **248**:1822–1829, 1973.
19. Corbin J, Sugden P, Lincoln T, Keely S. Compartmentalization of adenosine 3′:5′-monophosphate-dependent protein kinase in heart tissue. J Biol Chem **252**:3854–3861, 1977.

20. Palmer W, McPherson J, Walsh D. Critical controls in the evaluation of cAMP-dependent protein kinase activity ratios as indices of hormonal activation. J Biol Chem **255**:2663–2666, 1980.
21. Kemp B, Froscio M, Rogers A, Murray A. Multiple protein kinases from human lymphocytes: Identification of enzymes phosphorylating exogenous histone and casein. Biochem J **145**:241–249, 1975.
22. Piras M, Horenstein A, Piras R. Identification of multiple protein kinases in normal human lymphocytes. Enzyme **22**:219–229, 1977.
23. Chaplin D, Wedner H, Parker C. Protein phosphorylation in human peripheral blood lymphocytes. Subcellular distribution and partial characterization of adenosine 3′:5′-cyclic monophosphate-dependent protein kinase. Biochem J **182**:525–536, 1979.
24. Goueli S, Wilson M, Davis A, Ahmed K. Studies of prostatic cytosol protein kinases of the aging ACI rat. Mech Ageing Dev **23**:371–382, 1983.
25. Kumar R, Chiero L, Rodriguez D. Protein phosphotransferases from liver of ageing rats. Gerontology **30**: 37–48, 1984.
26. Lipson L, Bobrycki V, Bush M, Tietjen G, Yoon A. Insulin release in aging: Studies on adenylate cyclase, phosphodiesterase, and protein kinase in isolated islets of Langerhans of the rat. Endocrinology (Baltimore) **108**:620–624, 1981.
27. Lin T, Vinson N, Murono E, Osterman J, Nankin H. The aging Leydig Cell. VIII. Protein kinase activity. J Androl **4**:324–330, 1983.
28. Beck N, Park M. Maturation-related changes in catecholamine-dependent cyclic AMP and protein kinase in the rabbit myocardium. Dev Pharmacol Ther **3**: 129–138, 1981.
29. Kahn A, Meienhofer M-C, Guillouzo A, Cottreau D, Baffet G, Henry J, Dreyfus J-C. Modifications of phosphoproteins and protein kinases occurring with in vitro aging of cultured human cells. Gerontology **28**:360–370, 1982.
30. Tsisouras P, Kowatch M, Harman S. Age-related alterations of isolated rat leydig cell function: Gonadotropin receptors, adenosine 3′, 5′-monophosphate response, and testosterone secretion. Endocrinology (Baltimore) **105**:1400–1405, 1979.
31. Guarnieri T, Filburn C, Zitnik G, Roth G, Lakatta E. Contractile and biochemical correlates of β-adrenergic stimulation of the aged heart. Amer J Physiol **80**:H501–H508, 1980.
32. Singh D. Cyclic AMP-dependent protein kinase endogenous phosphorylation levels in young and adult rat hearts. Cell Mol Biol **28**:51–55, 1982.
33. Kraft C, Castleden C. The effect of aging on β-adrenoceptor-stimulated cyclic AMP formation in human lymphocytes. Clin Sci **60**:587–589, 1981.

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