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ISOPROTERENOL-INDUCED SELECTIVE PHOSPHORYLATIVE MODIFICATION IN VIVO OF RAT C6 GLIOMA CELL HISTONES

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SUMMARY The phosphorylative modification in vivo of histones after short-term (0 to 60 min) isoproterenol stimulation of confluent rat C6 glioma cell cultures has been investigated. Analysis of the phosphorylation patterns after the purification and separation of histones by SDS/polyacrylamide gel electrophoresis revealed significantly increased phosphorylation of histones H1-1 and H3 and a decrease of the phosphorylation of histones H1-3, H2A, and H2B. There was no apparent effect of isoproterenol on the net phosphorylation of histones H1-2 and H4. The data suggest an effect of isoproterenol on the phosphorylative modification of glioma cell histones via modulation of nuclear phosphorylating and dephosphorylating activities.

Phosphorylative modification of chromosomal proteins by nuclear protein kinases and phosphatases is believed to lead, in addition to structural changes, to functional changes as well (1,2). More specifically, nuclear proteins which participate in the regulation of gene expression may be subject to phosphorylative and functional modifications by nuclear protein kinases and phosphoprotein phosphatases.

Catecholamines, through the action of cAMP, have been shown to cause the induction of lactate dehydrogenase (3-5) and its mRNA (6) in C6 glioma cells. These cells, therefore, provide a suitable model for studying the role of nuclear proteins and their modification by nuclear protein kinases during the catecholamine-regulated expression of the lactate dehydrogenase gene. In previous studies we have provided evidence for the presence in glioma cell nuclei of two cAMP-independent protein kinases as well as one cAMP-dependent protein kinase which achieved a selective phosphorylative modification of several his-

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tones under cell-free in vitro conditions (7). In view of these findings we proceeded to examine whether isoproterenol could achieve the selective phosphorylation of rat C6 glioma cell histones in vivo, reflecting modulation of the activity of nuclear protein kinases. We report here a modulation of the degree of phosphorylation of several histones in C6 glioma cells as a consequence of isoproterenol stimulation.

MATERIALS AND METHODS (-) Isoproterenol bitartrate and all reagents of analytical grade were purchased from Sigma Chemical Company. Carrier-free $Na_2H^{32}PO_4$ (285 Ci/mg) was from Amersham/Searle or ICN Chemical Corporation.
Rat C6 glioma cells were obtained from the American Type Culture Collection and maintained at 38°C under 5% CO_2 in air in Ham's F-10 nutrient medium (GIBCO) supplemented with 10% fetal bovine serum or newborn calf serum, 50 U/ml of penicillin, and 50 µg/ml of streptomycin. Cells were used between 7 to 20 passages and were harvested at confluency either 10 days after passage into medium containing fetal bovine serum or 13 days after passage into medium containing newborn calf serum. Two days prior to an experiment, the medium was replaced with Ham's medium which did not contain serum. Fifteen hours prior to labeling with $Na_2H^{32}PO_4$, the serum-free medium was replaced with serum-free low-phosphate Ham's F-10 medium. Labeling of the cells was performed for 2 hours with 0.5 to 1 mCi/ml of $Na_2H^{32}PO_4$ in the low-phosphate medium. Isoproterenol was added for various time periods as indicated in the text.
To isolate total histone fraction, nuclei were prepared by homogenizing cells in 0.25 M sucrose, 3 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100. After 15 strokes in a glass-glass homogenizer, the homogenate was layered onto 0.5 M sucrose, 3 mM $MgCl_2$ and centrifuged for 1 hour at 100,000xg. The pelleted nuclei were rinsed with 0.25 M sucrose without disturbing the pellet. Nuclear nonhistone proteins were extracted by suspending the nuclei for 5 min at 2°C in 40 mM imidazole hydrochloride, 0.2 mM EDTA, 20 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.35 M NaCl, pH 7.0. The extract was centrifuged for 30 min at 100,000xg. The pellet was resuspended in the extraction buffer and extracted once more. The nucleohistone pellet was resuspended in 0.25 M HCl, and total histones were isolated according to Johns (8). Total histones were separated by discontinuous SDS/polyacrylamide slab gel electrophoresis according to Laemmli (9) on linear gradient 7.5 to 40% polyacrylamide gels. Individual histones were identified by co-migration of calf thymus histone standards. Quantitation of radioactivity in gel slices was performed as previously described (10).
To preferentially isolate histone H1, total histones were extracted directly from cells by the addition of 0.4 N H_2SO_4 to monolayers (11). After two acid extractions, histone H1 was separated from total acid-soluble histone by differential trichloroacetic acid precipitation (11). Histone H1 was subsequently subjected to electrophoresis on 18% polyacrylamide slab gels according to Laemmli (9) as modified by Bonner and Pollard (12). Histone H1 was identified by co-migration of highly purified calf thymus H1 (Boehringer Mannheim Corp.). Histone H1 bands were sliced from the gels, and the amido black-stained protein was eluted with 0.125 M Tris, 2% sodium dodecyl sulfate, pH 6.8. The amount of histone was determined by measuring the optical density at 600 nm as compared to the optical density of histone H1 standard (13). Eluted radioactivity was determined in RPI 3a70B scintillation cocktail in a Beckman LS9000 liquid scintillation spectrometer. The nomenclature of rat histone H1 subspecies is that of Gorka and Lawrence (14).

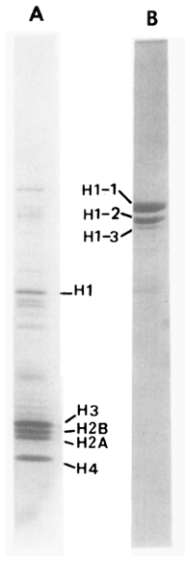

Figure 1. Separation of rat C6 glioma cell total histone fraction by gradient polyacrylamide gel electrophoresis (track A) and of histone fraction H1 on 18% polyacrylamide gels (track B). For experimental details see Methods.

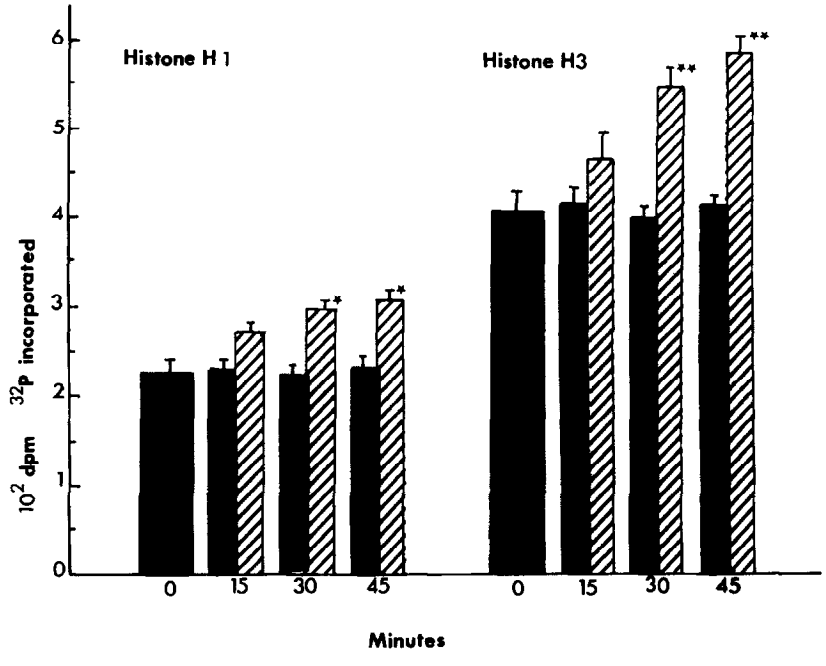

Figure 2. Time course of isoproterenol-mediated phosphorylation of glioma cell histones H1 and H3. Labeling with $Na_2H^{32}PO_4$, stimulation with 10 μM isoproterenol, isolation of histones by gradient polyacrylamide gel electrophoresis, and analysis of radioactivity in the stained histone bands were performed as described in Methods. Solid bars: untreated control cells; hatched bars: isoproterenol-stimulated cells. Brackets indicate the standard deviation of the mean (n = 5). * $p<0.01$; ** $p<0.005$.

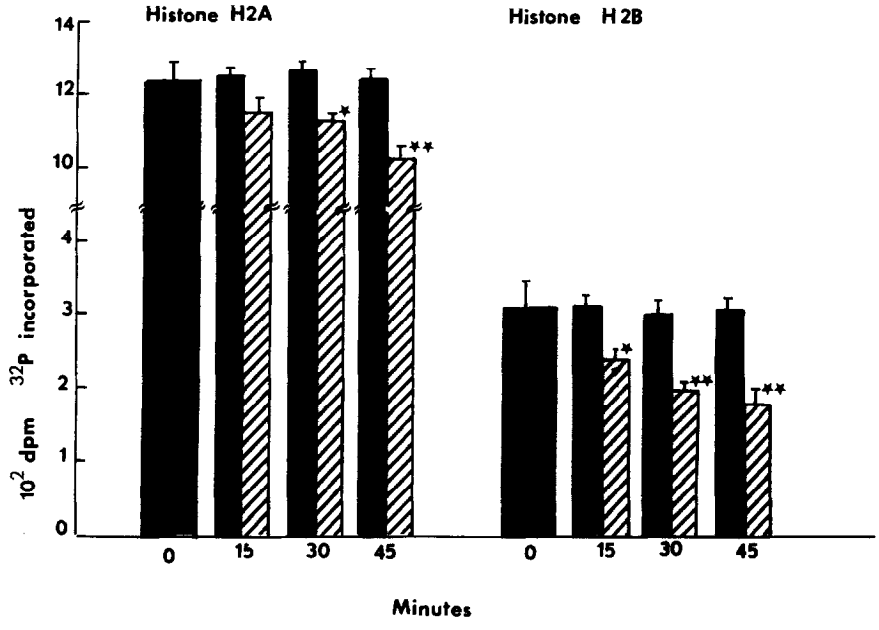

Figure 3. Time course of isoproterenol-mediated phosphorylative modification of glioma cell histones H2A and H2B. For experimental details see legend of Fig. 2.

RESULTS Electrophoresis of total histone fraction on gradient polyacrylamide gels results in the separation of all core histones, but it does not separate histone H1 into its subspecies (Fig. 1A). Separation of the histone subspecies is accomplished by electrophoresis on 18% polyacrylamide gels (Fig. 1B). Quantitation of [^{32}P]phosphate incorporation reveals detectable changes in the degree of *in vivo* phosphorylation of several histones after isoproterenol stimulation. Whereas isoproterenol causes a significant increase in the degree of phosphorylation of histone H1 and H3 (Fig. 2), the incorporation of ^{32}P-label into histones H2A and H2B is slightly but significantly decreased (Fig.3). The level of phosphorylation of histone H4 is not affected (data not shown). There is no significant change in [^{32}P]phosphate incorporation into histones in untreated control cultures, thus indicating that the observed phosphorylative modifications are due to the presence of isoproterenol.

TABLE I

Phosphorylative Modification of Histone H1 Subspecies in Isoproterenol-stimulated Rat C6 Glioma Cells

Histone	Specific Activity x 10^{-7}[1]		Specific Activity Ratio[2]	Percentage of Total Histone H1 Present in Subspecies	
	- Isoproterenol	+ Isoproterenol		- Isoproterenol	+ Isoproterenol
Total H1	3.25	4.45	1.37±0.13[3]	100	100
H1-1	3.36	5.12	1.52±0.15	70.8±2.7[3]	66.2±1.4
H1-2	3.30	3.83	1.16±0.12	22.4±2.9	25.5±2.4
H1-3	2.65	2.22	0.84±0.08	6.8±0.6	8.3±2.0

Rat C6 glioma cells were labeled with $Na_2H^{32}PO_4$ and then stimulated for 1 hour in the presence of 10 μM isoproterenol. Total histone fraction H1 was isolated, separated into its subspecies by electrophoresis, and ^{32}P radioactivity in each subspecies was determined. For experimental details see Methods.

[1] Specific activity = mol [^{32}P]phosphate incorporated/mol histone

[2] Ratio = Specific activity of stimulated cells/specific activity of control cells

[3] Values are given as means±standard deviation of the mean (n = 4).

Since histone fraction H1 consists of several subspecies (15), we next proceeded to determine whether or not the individual subspecies became phosphorylated in a selective, differential fashion. Whereas histone H1 can be separated into 5 subspecies by Amberlite IRC-50 chromatography, electrophoretic separation on SDS/polyacrylamide gels results in the separation of only 3 subspecies (15) (see Fig. 1B). Histone H1 was isolated from [^{32}P]phosphate-labeled cells without and with isoproterenol treatment and separated by electrophoresis into subspecies H1-1, H1-2, and H1-3. As shown in Table I, the extent of [^{32}P]phosphate incorporation into total histone H1 fraction is increased by about 37% in stimulated cells (see also Fig. 2). However, the increased phosphorylation is confined to the H1-1 subspecies. There is no statistically significant phosphorylative modification of histone H1-2 as determined by t-test analysis. Moreover, the degree of phosphorylation of histone H1-3 is slightly but significantly decreased in stimulated cells. Thus, it appears that the observed increased phosphorylation of total histone H1 fraction is due to a selective phosphorylation of the H1-1 subspecies. The phosphorylative modifications of histones H1-1 and H1-3 are not caused by differences in the amount of total extractable histone H1 between control and stimulated cells, since the extracted amounts are similar (data not shown) and since there are no quantitative variations in the amount of extracted histone subspecies (see Table I).

DISCUSSION We have demonstrated the *in vivo* phosphorylative modification of several core histones and of H1 subspecies as the result of isoproterenol stimulation. In addition to phosphorylation events of histones *in vivo* which occur during times of cellular replication (16), glucagon- or cAMP-mediated phosphorylation *in vivo* of histone H1 has been identified in rat liver (17-19) and Reuber H35 hepatoma cells (20). The increased phosphorylation of an H1-like protein in isoproterenol-stimulated rat C6 glioma cells has also been reported (21). The *in vivo* phosphorylation of histones H1-1 and H3, and the simultaneous dephosphorylation of histones H1-3, H2A, and H2B has to our knowledge not been reported so far.

The finding of increased phosphorylation of histones H1-1 and H3 but concomitant dephosphorylation of histones H1-3, H2A, and H2B suggests a complex control mechanism involving not only protein kinase(s) but phosphoprotein phosphatase(s) as well. Furthermore, work in progress in our laboratory to determine specific phosphorylation sites has provided preliminary evidence that some sites are phosphorylated concomitantly with the dephosphorylation of different sites within the same histone species. While this observation does not indicate that the modifications are occurring on the same molecule, it does indicate that the inability to detect a net change of phosphorylative modification of a particular histone species may not mean that the species is unaltered during stimulation. These modifications could involve activation of nuclear protein kinase(s) and phosphatase(s). Alternatively, isoproterenol-induced changes of chromatin structure may enable the interaction between histones and their modifying enzymes, thus leading to the modification.

In view of the relatively large quantities of histones in the nucleus, the observed levels of phosphorylative change must be considered relatively small. For instance, it can be calculated that isoproterenol stimulation involves transfer of about one molecule of phosphate per 10^7 molecules of histone H1. This low level of phosphorylation suggests a selective alteration of specific histones at certain chromatin loci rather than random phosphorylative modification of all chromatin-associated histones. Such selective modification might be involved in the regulation of transcriptional processes or structural changes of chromatin as they might occur, for example, during isoproterenol-mediated lactate dehydrogenase induction in rat C6 glioma cells.

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