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In Vitro Phosphorylation of Nuclear Proteins in Isolated Liver Nuclei from Rats Maintained in a Starvation State, Following Refeeding, and from Diabetic Rats with Insulin Injection

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斷食, 再給食 및 인슈린 投與 後에 쥐의 肝으로부터 분리된 세포핵의 핵단백질 인산화

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요 약

분리된 간세포핵을 [γ-32P] ATP와 5분간 37°C에서 배양한 뒤 Chromatin 단백질의 32P labelling을 관찰했다. 32P labelling의 pattern을 48시간 단식상태 및 48시간 굶긴 쥐에게 탄수화물 농도가 높은 먹이를 준 다음 12시간 경과한 쥐와 streptozotocin으로 당뇨병을 유발시킨 쥐에 인슈린을 투여한 뒤 6시간 경과한 쥐의 세포핵으로부터 추출한 핵단백질을 sodium dodecyl sulfate 전기영동으로 조사하였다.

48시간 굶은 쥐의 간세포핵의 경우에서는 0.14M NaCl에 용해하는 단백질의 인산화 수준은 정상적으로 먹이를 준 쥐에 비하여 분자량이 41,000~200,000 daltons 사이의 단백질에서는 감소되었다. 48시간 굶긴 쥐에서 본 인산화 감소를 12시간 내에 역전시켜 정상적으로 먹이를 준 쥐에서 관찰한 인산화 수준에까지 끌어 올렸다. 폐놀용해성 핵단백질의 인산화 수준은 분자량이 59,000 daltons 보다 큰 5단백질에서 48시간 굶긴 쥐에서 정상적으로 먹이를 준 쥐의 결과와 비교하였을 때 낮았다. 아울러 단식은 히스톤의 인산화도 감소되었다.

Streptozotocin으로 당뇨병을 유발시킨 쥐에게 인슈린 주사를 준 다음 6시간 경과한 쥐의 인산화 수준은 0.14M NaCl 용해성 핵단백질중 분자량이 130,000 daltons 단백질과 폐놀용해성 단백질의 경우에는 155,000 daltons 단백질에서 인슈린을 주지 않은 당뇨병을 가진 쥐와 비교하였을 때 증가를 보였다. 그러나 히스톤의 인산화 수준에는 큰 변화가 나타나지 않았다.

여기에 나타난 실험결과가 0.14M NaCl 용해성 핵단백질과 H_1 히스톤의 인산화와 탈인산화가 다른 핵단백질에 선행해서 일어난다는 가능성을 시사해 주고 있다. 그리고 인슈린 신호가 핵단백질의 인산화와 관련이 있는 반면 그루카곤(glucagon)은 핵단백질의 탈인산화와 상관관계가 있음은 매우 흥미있는 사실이다.

Introduction

A number of evidence have accumulted that the phosphorylation of nuclear proteins[1~7] may regulate the availability of gene sequences for transcription in eucaryotes. The early studies suggest that various hormones induce the phosphorylation of nulear proteins, in

particular, non-histone proteins and consequently influence gene regulation[8~14]. In liver, insulin promotes glycogenesis and lipogenesis through short term regulation of key enzymes such as acetyl carboxylase[15] and fatty acid synthetase complex[16] primarily by signalling the dephosphorylation of these enzymes. By contrast glucagon affects net phosphorylation. Recent studies[17~20] also indicate that insulin is the primary signal *in vivo* for initiating the synthesis of the lipogenic set of enzymes. In the other words, controled by insulin and glucagon is exerted though long term induction and repression of enzyme synthesis.

Since reversible phosphorylation of chromatin proteins may be prerequisite for gene transcription we decided to examine the state of protein phosphorylation in liver nuclei from rats in starvation and following high sucrose refeeding, or from rats injected with insulin, that is, under conditions in which enzymes are reversibly phosphorylated. With this approach, it will be of interest to test the possibility that insulin and glucagon may control both enzymes and chromatin protein phosphorylation through a concerted mechanism.

Materials and Methods

Unless stated otherwise here, all materials and methods employed for the experiments were same as those described in the previous publication.[21]

Phosphorylation experiment of nuclear proteins in isolated liver nuclei. The purified nuclei was washed with 2m*l* of 0.25M sucrose lmM $CaCl_2$ buffer twice. The phosphorylation of nuclear proteins was carried out by the slightly modified method of Thomson *et al*[22]. The washed nuclei pellet was suspended in incubation medium buffer containing 0.25M sucrose-40 mM Tris-HCl, pH8-20 mM $MgCl_2$-1mM $CaCl_2$-25mM NaCl. The incubation mixture for phosphorylation contained nuclei suspension equivalent to 10mg of nuclear protein and μCi [32P]-ATP in the final volume of 2m*l*. Immediately after the addition of [32P]-ATP, the suspension was incubated at 37°C by shaking for 5min. The phosphorylation reaction was stopped by adding 2m*l* of ice cold washing buffer which was consisted of phosphorylation reaction medium containing augmented concentration of APT to 5mM with cold ATP instead. The nuclei was pelleted by centrifugation at 100xG for 10min. The supernatant was decanted off and the pellet was washed with an additional 1m*l* of washing buffer before extraction of nuclear proteins. The nuclear sap proteins were extracted twice with 1m*l* of 0.14M NaCl. Histone fractions were obtained by washing the residue with 1m*l* of 0.25N HCl twice. Lipid extractions from the residue were performed subsequently with 2m*l* of chloroform: methanol (1 : 1, v/v) containing 0.2N HCl, 2m*l* of chloroform: methanol (2 : 1, v/v) containing 0.2N HCl and ether. Phenol soluble non-histone proteins were extracted with 2m*l* of phenol saturated with 0.1M Tris-HCl, pH 8.4, containing 0.01 methylenediamine tetraacetic acid and 0.14M 2-mercaptoethanol (buffer A) and 2m*l* of buffer A. The additional extractions were repeated twice with each 2m*l* of phenol saturated with buffer A.

Determination of 32P. After scanning the protein bands on the destained polyacrylamide gel, the gel was cut into 1mm thick slice by a gel slicer. Each sliced gel was transferred to a counting vial. One milliliter of NCS solubilizer (Amersham Product) was added to a counting vial. The vials were tightly capped and incubated at 50°C for 2 hours. Then the vials containing the sliced gel were cooled. The radioactivity of the solubilized gel was determined with 10m*l* of OCS counting solvent (Amersham Product) by a liquid scintillation spectrophotometer.

Results

Phosphorylation patterns of 0.14M NaCl soluble proteins. Figure 1 indicates that 0.14 M NaCl soluble proteins in the range of

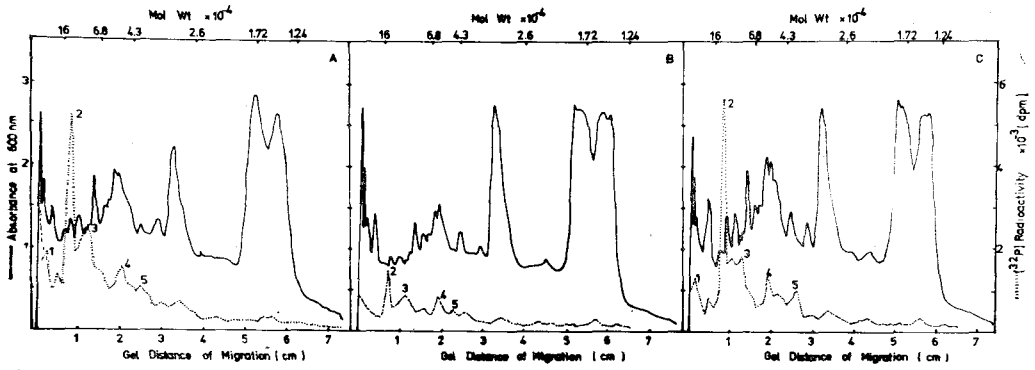

Fig. 1. Phosphorylation patterns of 0.14 M NaCl soluble proteins extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of rats with different nutritional states. The details of the experiments are given in the text. An aliquot of 50 μg protein was electrophorized on a tube- *Figure 1A:* normal feeding rats. *Figure 1B:* 48h starved rats. *Figure 1C:* 12h after refeeding of 48h starved rats. The molecular weights of the five major peaks with phosphorylation are: 1;200 kilodaltons, 2;130 kilodaltons, 3;100 kilodaltons, 4;53 kilodaltons, and 5;41 kilodaltons. The standard proteins used for estimation of molecular weight were: γ-globulin(human) (160,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen A (26,000 daltons), myoglobin (17,200 daltons) and cytochrome C (12,400 daltons).

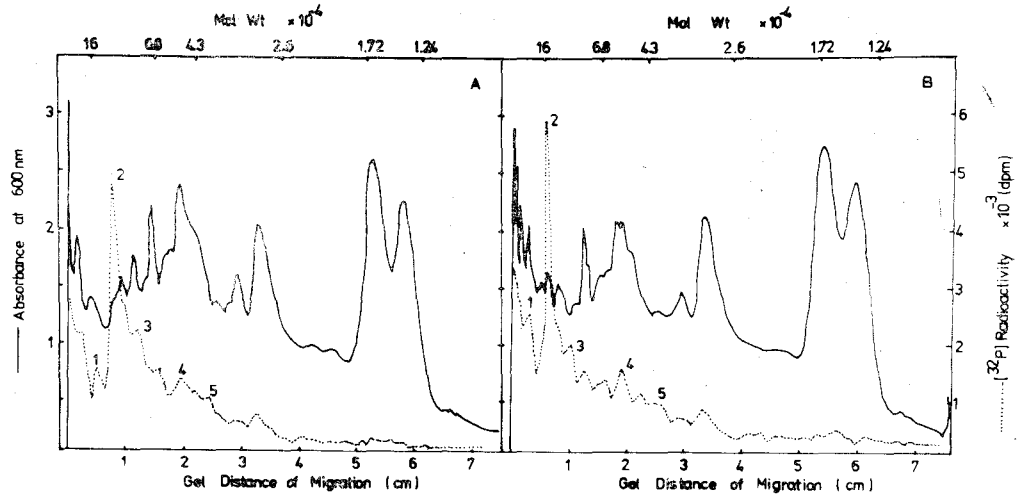

Fig. 2. Effect of insulin injection on the change of phosphorylation pattern of 0.14M NaCl soluble proteins extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of streptozotocin-diabetic rats. The details of the experiments are given in the text. An aliquot of 50μg protein was electrophorized on a tube. *Figure 2A:* 6h after saline injection into streptozotocin-diabetic rats. *Figure 2B:*6h after insulin injection into streptoztocin-diabetic rats. The molecular weights of the five major peaks with phosphorylation are: 1;200 kilodaltons, 2;130 kilodaltons, 3;100 kilodaltons, and 5;41 kilodaltons.

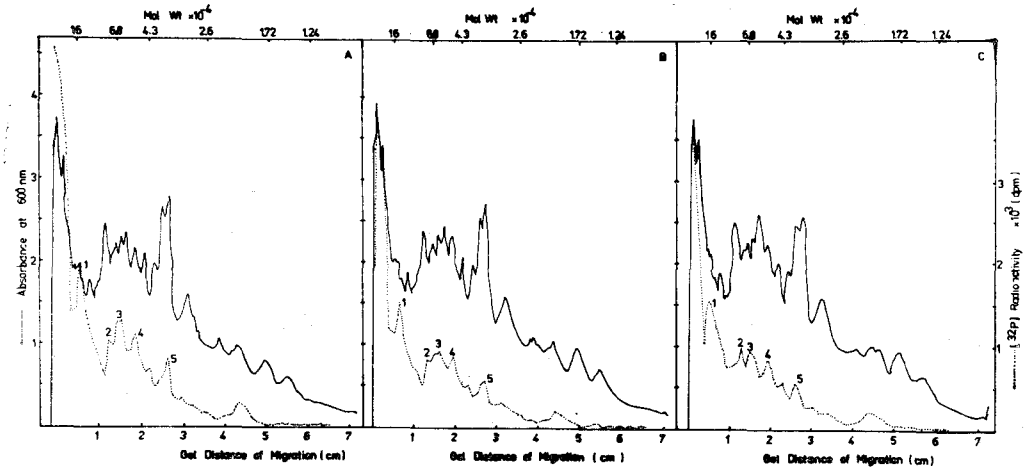

Fig. 3. Phosphorylation patterns of phenol soluble non-histone proteins extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of rats with different nutritional states. The details of the experiments are given in the text. An aliquot of 50μg protein was electrophorized on a tube. *Figure 3A:* normal feeding rats. *Figure 3B:* 48h starved rats. *Figure 3C:* 12h after refeeding of 48h starved rats. The molecular weights of the five major peaks with phosphorylation are: 1;155 kilodaltons, 2;100 kilodaltons, 3;65 kilodaltons, 4;59 kilodaltons, and 5;41 kilodaltons.

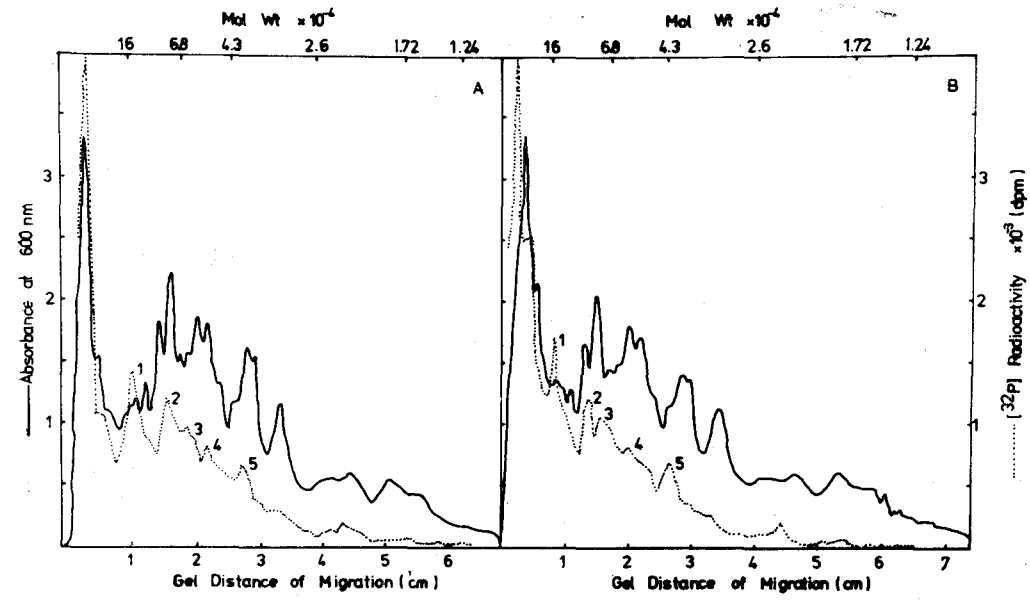

Fig. 4. Effect of insulin injection on the change of phosphorylation pattern of phenol soluble non-histone proteins extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of streptozotocin-diabetic rats. The details of the experiments are given in the text. An aliquot of 50μg protein was electrophorized on a tube. *Figure 4A:* 6h after saline injection into streptozotocin-diabetic rats. *Figure 4B:* 6h after insulin injection into streptozotocin-diabetic rats. The molecular weights of the five major peaks with phosphorylation are: 1;155 kilodaltons, 2;100 kilodaltons, 3;65 kilodaltons, 4;59 kilodaltons, and 41 kilodaltons.

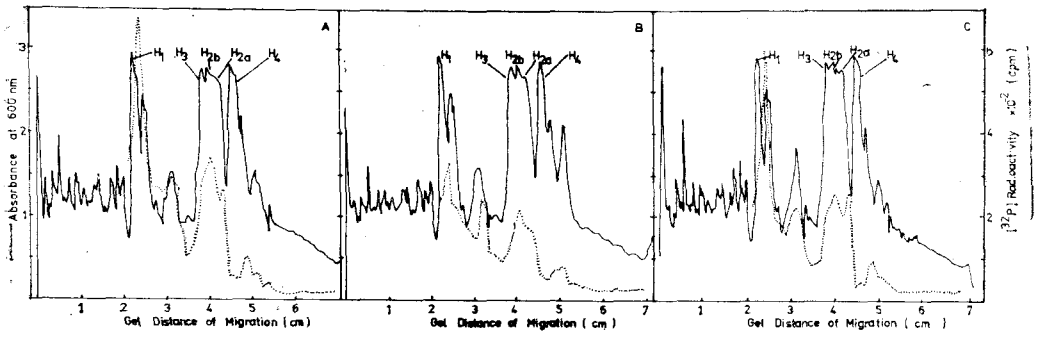

Fig. 5. Phosphorylation patterns of histone fractions extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of rats with different nutritional states. The details of the experiments are given in the text. An aliquot of 100μg protein was electrophorized on a tube. *Figure 5A:* normal feeding rats. *Figure 5B:* 48h starved rats. *Figure 3C:* 12h after refeeding of 48h starved rats.

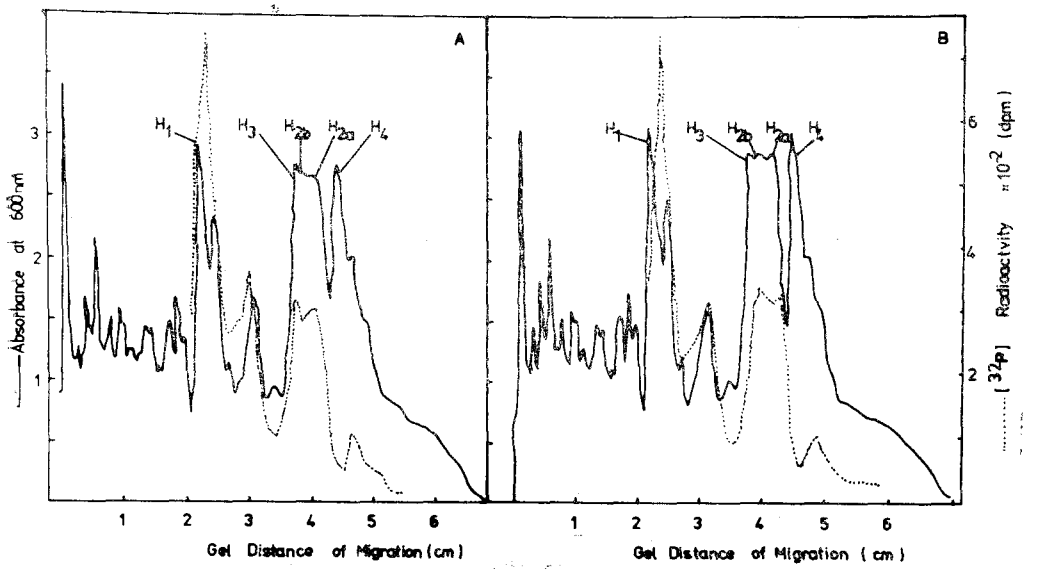

Fig. 6. Effect of insulin injection on the change of phosphorylation pattern of histone fractions extracted from nuclei incubated in phosphorylation medium containing [γ-32P]-ATP of streptozotocin-diabeticrats. The details of the experiments are given in the text. An aliquot of 100μg protein was electrophorized on a tube. *Figure 6A:* 6h after saline injection into streptozotocin-diabetic rats. *Figure 6B:* 6h after insulin injection into streptozotocin-diabetic rats.

molecular weights between 41,000 and 200,000 daltons were sensitive to phosphorylation. With 48 h starved rats the level of phosphorylation for 0.14M NaCl soluble proteins extracted from liver nuclei was considerably decreased relative to normal controls. However, it can be noticed that the decrease of phosphorylation level may be attributable to the decreased concentration of phosphorylation sensitive proteins. Refeeding the starved rats reversed the change of phosphorylation pattern and protein concentrations over 12 hours. The electrophoretic patterns of 0.14M NaCl soluble proteins obtained from phosphorylation experiment is not similar to the results reported previously[21]. The dissimilarity may be due to the involvement of the additional washings of purified nuclei with the phosphorylation incubation buffer before and after the incubation of the purified nuclei at 37°C in phosphorylation experiment.

The experiment with insulin injection into fed streptozotocin diabetic rats showed the tendency to increase phosphorylation level of a protein with molecular weight of approximately 130,000 daltons which is most sensitive to phospholation in 0.14M NaCl soluble protein fractions (Fig. 2). Unlike Fig. 1, there was no noticable change in the relative concentrations of 0.14M NaCl soluble proteins between diabetic rats and diabetic rats with insulin injection 6 hours before sacrifice of animals.

Phlosphorylation patterns of phenol soluble non-histone proteins. Figure 3 shows that 48h starvation of rats decreased the levels of phosphorylation for five non-histone proteins with molecular weights of 41, 59, 65, 100, and 155 kilodaltons, respectively. Unlike the case of 0.14M NaCl soluble proteins, refeeding the starved rats did not reverse the change of phosphorylation pattern over 12 hours.

Insulin injection into fed streptozotocin diabetic rats had no significant effect on the phosphorylation of phenol soluble non-histone proteins with lower molecular weight than 155,000 daltons under the conditions employed here (Fig. 4). This result is very similar to that of refeedings 48h starved rats as observed in Fig. 3.

Phosphorylation patterns of histones. As observed in the experiment with 0.14M NaCl soluble proteins starvation caused the noticable decrease in 32P incoporation into five major histones; H_1, H_4, H_{2b}, H_{2a}, and H_4. The drastic change of phosphorylation level by starvation was observed with H_1 histone. Refeeding starved rats almost reversed the change in the level of phosphorylation with H_1 over 12 hours. However, the degree of increased phosphorylation by refeeding was much less with the rest of histones; H_3, H_{2b}, H_{2a}, and H_4 as compared with that of H_1 (Fig. 5).

Unlike the results obtained from nutritional state changes, the phosphorylation level of histones was not significantly varied with insulin injection into diabetic rats 6 hours before sacrifice of animals relative to diabetic controls (Fig. 6).

Discussion

The effect of insulin or glucagon on phosphorylation of histones has been intensively investigated with isolated perfused liver *in vitro*[23] or *in vivo*[24]. It has been found that these hormones cause a marked increase in the phosphorylation of a specific amino acid residue of histones. Turkington and Riddle[9] have shown that insulin stimulates nuclear protein phosphorylation significantly in mouse mammary epithelial cell culture. To date there has not been any report concerning the effect of elevation of insulin or glucagon concentration in liver cell caused by change of nutritional states of animal on phosphorylation of nuclear proteins in the isolated nucleus.

The results presented here demonstrate that phosphorylation pattern of three major nuclear proteins is not qualitatively, but quantitatively in response to the variation of nutritional states when analyzed by polyacrylamide gel

electrophoresis in the presence of sodium dodecyl sulfate. The levels of phosphorylation of nuclear sap proteins appear to be considerably dependent upon the concentration of phosphorylation-sensitive proteins while those of phenol soluble non-histone proteins and histones reflect the phosphorylation state of the nuclear proteins.

It is of interest to find that refeeding starved rats for 12h did not have the comparable reversal of phosphorylation level of phenol soluble non-histone proteins and histones except H_1 to the phosphorylation level of normal controls. This finding suggests the possibility that it may take more than 12 hours for these nuclear proteins to reach the same or higher level of phosphorylation as observed in normal feeding controls. Also, the results indicate that the phosphorylation of 0.14M NaCl soluble proteins and H_1 histone may precede that of other chromatin associated nuclear proteins.

In case that the effect of insulin injection into diabetic rats on endogenous phosphorylation capability of isolated liver nuclei was examined, the phosphorylation patterns shown here indicate that insulin acts as a mediator to change the phosphorylation capability of specific proteins in isolated nuclei among 0.14M NaCl soluble proteins and phenol soluble non-histone proteins while the phosphorylation of histones is little affected. The results are somewhat different from the previous findings that insulin causes significantly increased phosphorylation of histones in *in vivo* studies[24] and mouse mammary epithelical cells in organ culture [9]. The discrepancy may be due to involvement of cytosolic factors in phosphorylation of nuclear proteins with both *in vivo* and cell culture studies.

Less marked increase in phosphorylation of nuclear proteins (Fig. 4 and Fig. 6) may be associated with short period (6h) of insulin administration not enough to activate phosphorylation system of rat liver nuclei. Turkington and Riddle[9] reported that incorporation of 32P into acid soluble nuclear proteins is significantly stimulated 8h after the addition of insulin to the cell culture medium.

Perhaps most interesting results obtained from phosphorylation studies are related to the state of phosphorylation-dephosphorylation of nuclear proteins modulated by glucagon and insulin signals. In other words, the switch from the gluconeogenic state in starvation to the lipogenic state by refeeding of the liver (insulin signal) was correlated to phosphorylation of nuclear proteins while glucagon signal dephosphorylated nuclear proteins. These results are opposite to the findings with cytosolic enzymes related to gluconeogenic and lipogenic metabolism on the basis of the state of phosphorylation-dephosphorylation of proteins modulated by glucagon and insulin signals.

Acknowledgments

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Abstract

Labelling of chromatin proteins with 32P was observed after incubating isolated liver nuclei with [γ-32P] ATP for 5 minutes at 37°C. The pattern of labelling with 32P was examined on SDS polyacrylamide gel electrophoresis with nuclei from rats maintained in a starvation state for 48 hours, following refeeding for 12 hours; and from fed streptozotocin-diabetic rats with insulin injection 6 hours before sacrifice.

With 48h starved rat liver nuclei the level of phosphorylation for 0.14M NaCl soluble proteins was decreased in the molecular weights between 41,000 and 200,000 daltons relative to normal controls. Refeeding the starved rats reversed the change of phosphorylation pattern over 12 hour. The level of

phosphorylation for five phenol soluble non-histone proteins with molecular weights above 59,000 daltons was somewhat decreased with 48h starved rat liver nuclei as compared with that of normal controls. Starvation also decreased the phosphorylation level of major histones in relation to normal controls. The experiment with insulin injection into fed streptozotocin-diabetic rats showed the tendency to increase phosphorylation of 0.14M NaCl soluble proteins (130,000 dalton protein) and phenol soluble non-histone proteins (155,000 dalton protein). The phosphorylation level of histones appeared to be invariant under the experimental conditoins employed here.

These results suggest the possibility that the phosphorylation and dephosphorylation of 0.14M NaCl soluble proteins and H_1 histone precede those of other chromatin associated nuclear proteins, It is of interest to find that insulin signal was correlated to phosphorylation of nuclear proteins while glucagon signal dephosphorylated nuclear proteins.

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