Nuclear Proteins in the Heart of the Cardiomyopathic Syrian Hamster

Phosphorylation of Phenol-Soluble Nonhistone Proteins

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SUMMARY We examined incorporation of $^{32}$P into phenol-soluble nonhistone nuclear acidic proteins (NHNP) obtained from myolytic, hypertrophic, and failing phases of hamster cardiomyopathy. NHNP from these dystrophic hamster hearts were phosphorylated much less than their controls, despite a 9-fold increase in uptake of $^{32}$P into their nuclei. After fractionation of NHNP by isoelectrofocusing polyacrylamide gel electrophoresis, two major fractions focusing from pH 6.0 to 6.2 and 6.6 to 6.8 were highly phosphorylated in both the control and dystrophic hearts. The latter fraction was much more phosphorylated in the control. Two fractions of NHNP focusing at pH 4.9 and 5.1 were more highly phosphorylated in the dystrophic hearts than in the controls. Autoradiographs obtained from the two-dimensional polyacrylamide slab gel electrophoresis showed that two proteins (pH 4.9 and 5.1; mol wt 25,000 and 60,000, respectively) were highly phosphorylated in the dystrophic heart. There was no detectable phosphorylation of these proteins in the controls. These changes in the phosphorylation of cardiac NHNP may be important in determining the alteration of gene expression in hamster cardiomyopathy.

HISTONE and nonhistone chromatin proteins or nonhistone nuclear proteins (NHNP) are known to be associated with DNA in eukaryotic cells. Histones can be fractionated into five major components which show a striking similarity in all species and in all tissues of an organism. NHNP are highly heterogeneous, exhibiting some degree of quantitative and qualitative variations in different tissues. This class of proteins has been implicated in the control of differential gene expression in higher organisms. There is evidence that changes in genetic activity are accompanied by alterations in NHNP. Several specific NHNP have been reported to be altered under different physiological states such as cell differentiation and hormone treatment. There also is considerable evidence that phosphorylation of NHNP is coupled to gene activation. Recent studies by Kleinsmith and co-workers, and others indicate that the phosphorylation and dephosphorylation of NHNP may be important in the regulation of gene transcription. Specific phosphorylation and dephosphorylation of NHNP have been shown to occur in differential gene activation by aldosterone and cyclic AMP.

The cardiomyopathic hamster is a genetic model of myocardial disease. As the cardiomyopathy develops naturally, without surgical intervention, this paradigm has proved useful in the study of the biochemical events associated with cardiac hypertrophy and failure. Nair and co-workers have shown an increase in DNA-dependent RNA polymerase activity in nuclear preparations derived from the hearts of cardiomyopathic hamsters in the "myolytic" or "necrotic" phase of the disease.

There is no report examining the metabolism of nuclear proteins in cardiomyopathic hamsters. In an earlier study, we demonstrated alterations in NHNP composition of myocardium from patients with muscular subaortic stenosis as compared to those with infundibular hypertrophy or controls. In a previous report, we showed that there are striking differences in NHNP between the hearts of cardiomyopathic hamsters and controls. This report deals with the covalent modification of heart NHNP at different stages of the development of the cardiomyopathy.

Methods

All chemicals and organic solvents used in these studies were of reagent grade. N,N,N',N'-tetramethylethylenediamine, acrylamide, and N,N'-methylene bisacrylamide were purchased from Eastman Organic Chemicals. Sucrose and urea were from Schwarz/Mann.

Animals

Cardiomyopathic (Bio 53.58) and control (RB) Syrian hamsters of both sexes, 35–270 days of age, were used in these experiments, as described in a previous report. Radioactive phosphorus acid ($^{32}$P-carrier free) was purchased from New England Nuclear. Each hamster was given 2.5 mCi $^{32}$P, intraperitoneally, and was killed by decapitation 100 minutes after injection.

Fractionation of Nuclear Proteins

Isolation of nuclei was carried out as described previously. Nuclei recovered from 2.2 m sucrose-10 mM Tris-HCl (pH 8.0)-3 mM MgCl$_2$-0.1 mM phenylmethylsulfonfylfluoride (PMSF) were resuspended once in medium A and pelleted again by low-speed centrifugation. Nuclei
were then suspended in 0.14 M NaCl to extract all the nucleoplasmic proteins. This fraction was defined as the 0.14 M NaCl-soluble fraction. The proteins that were associated with DNA and not removed by 0.14 M NaCl were subsequently fractionated into 0.25 M HCl-soluble proteins and HCl-insoluble proteins. In this study, the acid-insoluble proteins were solubilized in phenol and defined as phenol-soluble NHNP, as described previously.26,27 The phenol extraction of proteins provides a good method for preventing any possible contamination of protein fractions by nucleic acids. The incorporation of $^{32}$P into NHNP is therefore regarded as protein phosphorylation. The acid-soluble proteins which were composed mainly of histone are described elsewhere.30

**Polyacrylamide Gel Electrophoresis**

NHNP were fractionated by isoelectrofocusing in the first dimension and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the second dimension.26,31 Following electrophoresis, the slab gels were fixed with 10% trichloroacetic acid (TCA) and stained with 0.1% Coomassie blue in 10% acetic acid for 4 hours. Destaining of the gels was carried out in 25% ethanol and 10% acetic acid. The gels were then stored in 7% acetic acid.

The two-dimensional slab gels, after destaining, were placed on Whatman 3-MM paper and were sealed with Saran wrap. The gels were then dried under vacuum overnight.

The $^{32}$P-labeled NHNP were detected by autoradiography using Kodak x-ray film (NS-54T). The gels were exposed to x-ray film for at least 2 weeks for heart NHNP, and 2–3 days for liver NHNP.

**Determination of Phosphate Content in Heart Tissue**

Heart homogenate (0.5 ml) was precipitated by 5% perchloric acid. Following low-speed centrifugation (1000 g for 5 minutes), the phosphate content of the supernatant extract was determined by the method of Martin and Doty.32 The specific activity of phosphate was determined and expressed as counts per minute per μmole of phosphate.

**Determination of Protein, Molecular Weights, and pH Gradients**

Protein determination was carried out by the method of Lowry et al.,33 using bovine serum albumin (BSA) as the standard. NHNP, which were in buffers containing 8.0 M urea-20 mM Tris-HCl (pH 8.4)-20 mM glycine-6 mM 2-$\beta$-mercaptoethanol, were precipitated by 5% TCA. It was necessary to remove the buffers from the proteins to prevent interference at $A_{260}$ nm. Molecular weights of NHNP were estimated by using known protein markers, e.g., human carbonic anhydrase (29,000), ovalbumin (45,000), BSA (67,000), and phosphorylase A (91,000).

The pH gradients of the isoelectrofocusing gels were determined by the method of MacGillivray and Rickwood.34

### Results

**Phosphorylation of NHNP in Three Different Stages of Hamster Cardiomyopathy**

Inorganic ($^{32}$P) phosphate was given to three groups of hamsters at different stages of the cardiomyopathy and their controls, as described in Methods. Heart nuclei were isolated and NHNP were obtained. As shown in Figure 1, the uptake of $^{32}$P into the nuclear suspension increased to 495% in the myolytic phase and 885% and 910% of the controls in the hypertrophic and failing phases, respectively. However, following the extraction of nucleic acids, phospholipids, and histone, which contained most of the $^{32}$P, it was found that phosphorylation of NHNP decreased to 74% of the control in the myolytic phase and to 60% and 56% in the hypertrophic and failing phases, respectively.

**Incorporation of $^{32}$P into Nuclear Proteins of Cardiomyopathic Hamsters in the Failing Phase**

We examined the nuclear proteins of failing ventricles in more detail. Nuclei were isolated from ventricles which...
were pulse-labeled with $^{32}$P and were fractionated into 0.14 M NaCl-soluble, 0.25 M HCl-soluble, and phenol-soluble proteins (i.e., NHNP). As shown in Table 1, the incorporation of $^{32}$P into the sodium chloride-soluble fraction increased 25-fold as compared to the control. The phosphorylation of acid-soluble proteins composed of more than 90% histone increased 1.9-fold. However, the phosphorylation of NHNP decreased significantly to 56% of control values.

We also examined the incorporation of $^{32}$P into the acid-insoluble fraction of whole ventricular homogenates (nuclei and cytoplasm) which were solubilized in phenol. As shown in Table 2, there were no apparent differences in protein phosphorylation between the control and the cardiomyopathic acid-insoluble proteins of heart homogenates. These proteins were much less phosphorylated than the NHNP. The phosphorylation of liver NHNP in both groups was found to be similar.

The specific activity of phosphate in the heart homogenate from both hypertrophic and failing phases of cardiomyopathy was also determined. As shown in Table 3, there was no apparent difference in specific activity of phosphate between hearts in the hypertrophic phase of the disease and their respective controls. There appeared to be a decrease of 33% in the specific activity of failing hearts. However, as shown in Table 1, the incorporation of $^{32}$P into the nuclear suspension, at this stage, increased to 862% of the control values.

**Fractionation of NHNP by One-Dimensional Polyacrylamide Gel Electrophoresis**

NHNP labeled with inorganic ($^{32}$P)phosphate in vivo from the failing phase of cardiomyopathy and its control were fractionated by isoelectrofocusing polyacrylamide gel electrophoresis. As shown in Figure 2, several NHNP fractions of the dystrophic hearts were less phosphorylated than the controls. Two major fractions of NHNP focusing from pH 6.0 to 6.2 and pH 6.6 to 6.8 were highly phosphorylated in both the control and dystrophic hearts. The latter fraction was much more highly phosphorylated in the control. There were two fractions of NHNP at pH 4.9 and

![Figure 2](https://example.com/figure2.png)
5.1 in the dystrophic hearts that exhibited a significant increase in phosphorylation.

Identification of Phosphorylated NHNP by Two-Dimensional Polyacrylamide Gel Electrophoresis

NHNP from the hearts of $^{32}$P-labeled hamsters in the myolytic, hypertrophic, and failing phases of the cardiomyopathy were fractionated by two-dimensional polyacrylamide gel electrophoresis. As shown in Figure 3 (M, H, F), there was a greater amount of radioactivity incorporated in most of the phosphorylated NHNP of control hamsters, as compared to cardiomyopathic hamsters. However, two striking phosphorylated cardiac NHNP were found in all three phases of the cardiomyopathy examined. These two proteins were found even in young hamsters with little overt histological evidence of heart disease. They were characterized by isoelectric points of 5.1 ($A_1$) and 4.9 ($A_2$) and molecular weights of 60,000 and 25,000, respectively. We demonstrated in the preceding paper$^{26}$ that there were striking differences in NHNP between the control and the dystrophic hearts. Protein ($A_1$) appears to correspond to protein ($P_2$) in our previous report.$^{26}$ Although this protein was reduced in the dystrophic heart, it was highly phosphorylated.

We also analyzed acid-insoluble proteins of whole heart homogenate, which were soluble in phenol, and found them to be identical in both groups. However, the two proteins that were found to be highly phosphorylated only in the nuclei of the cardiomyopathic hamsters were present in the heart homogenates of both groups (Fig. 4). These two "whole heart" proteins appeared to incorporate less radioactive phosphorus than their nuclear counterparts.

$^{32}$P-labeled NHNP which were isolated from liver were also exposed to x-ray film. Most liver NHNP were found to be highly phosphorylated, and there were no striking differences between myopathic and control hamsters (Fig.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3** Autoradiographs of NHNP fractionated by two-dimensional polyacrylamide gel electrophoresis. In vivo $^{32}$P-labeled heart NHNP isolated from myolytic (M), hypertrophic (H), and failing (F) phases of cardiomyopathy were fractionated by slab gel electrophoresis. The stained gels were dried and exposed to x-ray film. The lower plates represent three different phases of cardiomyopathy and the upper plates represent their respective controls.
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5). NHNP A₂, which was highly phosphorylated in myopathic heart nuclei, was not detected in the liver.

Discussion

In a previous paper we demonstrated that the high resolution of NHNP achieved by two-dimensional polyacrylamide gel electrophoresis could reveal striking differences in these proteins between control and myopathic hearts as early as the myolytic phase of the cardiomyopathy. We have also shown that these differences reflect changes in the biochemical composition of heart muscle cell nuclei. This report deals with the phosphorylation of heart NHNP and further substantiates the hypothesis that chromatin proteins are involved in the development of cardiomyopathy.

We found that the phosphorylation of cardiac NHNP decreased significantly in cardiomyopathy. The decrease in NHNP phosphorylation probably was not due to the pool size of phosphate for the following reasons: (1) the uptake of ³²P into the nuclear suspension of the myopathic hearts was approximately 9-fold greater than the control (Table 1); (2) the incorporation of ³²P into acid-insoluble proteins that were obtained from heart homogenates of the dystrophic and control hamsters was identical (Table 2); and (3) the incorporation of ³²P into liver NHNP in the cardiomyopathic groups was similar to the control (Table 2).

Although total cardiac NHNP phosphorylation was low in cardiomyopathy, as compared to the controls, two NHNP identified by two-dimensional polyacrylamide gel electrophoresis were highly phosphorylated in dystrophic hearts. These two phosphorylated proteins, identified by their pH values of 4.9 and 5.1, molecular weights of 25,000 and 60,000, respectively, were highly specific for the dystrophic hearts and were found at all stages of cardiomyopathy including the earliest phase of myolysis.

These two NHNP were also found although apparently with less incorporated radioactive phosphorus, in the phenol-soluble proteins derived from the acid-insoluble whole heart homogenates of both control and dystrophic hamsters.

In addition to the phosphorylation of two specific NHNP, we also found an increase in the phosphorylation of histone HI in dystrophic hearts. Allfrey and his coworkers have demonstrated that one of the most striking effects of histone HI is a stimulation of the phosphorylation of NHNP with molecular weights ranging from 30,000 to 60,000. The specific relationship between histone HI phosphorylation and the two specific phospho-

![Figure 4](image-url)
 Autoradiography of liver NHNP. Liver NHNP from the control (N) and failing (F) hamsters were fractionated by slab gel electrophoresis.

Phosphorylated NHNP reported in this study is, of course, unknown.

The electrophoretic characteristics of the protein focusing at pH 4.9 with a molecular weight of 25,000 were similar to one light chain of myosin. A phosphorylated chromatin acidic protein with a molecular weight similar to our second protein (mol wt 60,000) was recently described by Johnson and Hadden. They reported that phosphorylation of the protein was stimulated by cholinergic agents and was cyclic nucleotide dependent. It is well known that norepinephrine is a potent stimulus for cyclic AMP production in the heart. Sole et al. found a considerable increase in norepinephrine turnover as early as the myolytic phase of hamster cardiomyopathy. Furthermore, Nair and co-workers described a similar increase in myocardial adenyl cyclase activity. Administration of pharmacological doses of isoproterenol to normal rats results in both cardiac hypertrophy and an increase in type I cyclic AMP-dependent protein kinase in the heart. This kinase has been associated with the modulation of specific gene expression. In this setting, it is of interest to note that the hearts of juvenile dystrophic hamsters are extremely susceptible to catecholamine-induced damage. Adrenergic blockade, on the other hand, reportedly has a favorable influence on the course of cardiomyopathy.

We conclude that the phosphorylation of cardiac NHNP may be important in the alteration of genetic expression in hamster cardiomyopathy. Furthermore, if dystrophic hamster myocardium is indeed supersensitive to catecholamines, there may be a relationship between the phosphorylation of these nuclear proteins and the increases in both cardiac norepinephrine turnover and adenyl cyclase activity characteristic of this disease.

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