ADP-ribosylation of nuclear proteins is increased by phenobarbital

Identification of the ADP-ribosylated histone fractions in rat liver nuclei

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Changes in the ADP-ribosylation of total proteins and purified histones of rat liver nuclei after phenobarbital treatment (80 mg/kg, 24 h) have been studied. The [^{32}P]NAD incorporation into total trichloroacetic acid precipitated proteins, in histone H1 and in core histones was evaluated, the specific radioactivities increasing 150, 40 and 8%, respectively. Histones H1 and H2B were the best ADP-ribose acceptors. Histone H4 did not show any ^{32}P incorporation, as revealed by autoradiography after SDS-PAGE of the purified histones, in either the control or phenobarbital treated rats. Possible involvement of ADP-ribosylation of nuclear proteins in the adaptative response of liver to phenobarbital is discussed.

Nuclear protein *Histone* *ADP-ribosylation* *Phenobarbital stimulation*

1. INTRODUCTION

The supramolecular organization of the chromatin is an important factor for the regulation of the genetic activity in mammalian cells. The blockade of the positive charges of the N-terminal residues of the histone by acetylation, phosphorylation, methylation or ADP-ribosylation, has been demonstrated to play a fundamental role in gene regulation by affecting DNA/histone interactions, particularly during cell differentiation (reviews [1,2]).

In spite of the known phenotypic stability of somatic cells, hepatocytes have a highly developed capacity to respond to endogenous and exogenous stimuli, displaying induction and repression mechanisms in adaptation to variations in hormone levels and nutritional intake as well as to injury by xenobiotics [3–5].

Among a multitude of chemical agents which affect protein synthesis in the liver, phenobarbital (PB) is known to induce a strong adaptative response characterized by marked hypertrophy and induction of the cytochrome P-450 monooxygenases. This phenotypic shift is the result of complex mechanisms involving both translational and transcriptional regulations [6]. We have previously demonstrated that the activity of ADP-ribosyltransferase is markedly increased in rat liver nuclei following PB administration, paralleling the increases in microsomal cytochrome P-450 monooxygenases [7].

This enzyme system catalyzes the transfer of the ADP-ribosyl moiety of NAD into nuclear proteins, giving rise to the covalent attachment of mono, oligo or poly(ADP-R) to the amino or carboxylic groups of amino acid residues in the protein acceptor molecules [8]. The histones are known to be major acceptors of ADP-R residues both in vitro and in vivo [9,10], this covalent modification being postulated to regulate the activity of the chromatin [11].

To investigate whether the increase in ADP-ribosyltransferase activity observed after treatment

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of rats with PB [7] results in a modification of the nuclear histones, which might in turn be involved in the adaptive response of liver, we performed a quantitative study of the net ADP-ribosylation of total nuclear proteins and of purified histone fractions from the livers of rats undergoing PB treatment.

2. MATERIALS AND METHODS

Male Wistar rats aged 3–3.5 months from the Gulbenkian Institute Animal House were used throughout this investigation. The animals were starved for 24 h, receiving water ad libitum before they were weighed and killed by decapitation. Control and experimental animals were kept under the same experimental conditions.

Rats treated with PB received 80 mg/kg body wt intragastrically in aqueous solution (16 mg/ml) 24 h before being killed.

2.1. *Cell fractionation*

Nuclei were isolated by differential centrifugation of the fresh liver homogenate in 2.2 M sucrose and 15 mM $MgCl_2$ as described in [7] except that 0.5 mM phenylmethylsulphonyl fluoride (PMSF) was added to the buffers to prevent proteolytic degradation [12].

2.2. *ADP-ribosylation reaction in nuclear suspensions*

[^{32}P]NAD incorporation was performed according to Rickwood et al. [13] and Okayama et al. [14] with minor modifications. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 30 mM $MgCl_2$, 60 mM KCl, 4 mM 2-mercaptoethanol, 4 mM NaF, 2.5 μmol adenylate [^{32}P]NAD^+ (50 Ci/mmol). Rat liver nuclei were added (125 μg protein) in a total volume of 25 ml. After incubation at 37°C for 7.5 min the reaction was stopped by transferring the samples to an ice bath.

Radioactivity incorporated into the macromolecules was measured by precipitating 50–100 μl aliquot samples by the addition of ice-cold trichloroacetic acid to a final concentration of 10%. The acid insoluble material was collected on Whatman GFC glass-fibre discs by filtration under negative pressure [13], washed three times with 10% trichloroacetic acid and three times with ethanol. After drying the radioactivity was measured by liquid scintillation counting.

2.3. *Extraction and electrophoresis of the histones*

Histones were isolated from the pre-incubated nuclei according to Johns [15] modified by Palau and Daban [16].

The extraction procedure was performed at 4°C. Nuclear suspensions were treated with 5% $HClO_4$ and the histone H1 fraction purified from the supernatant. The $HClO_4$ pellet was used to obtain the core histones by extraction with 0.25 N HCl. The purified histone fractions were precipitated, washed with acetone at −20°C and dried.

The specific radioactivity of both the total proteins precipitated from the nuclear suspensions and the purified histone fractions was calculated by ^{32}P liquid scintillation counting and protein determination according to Lowry et al. [17].

The purified histone fractions were further analysed by 15% PAGE in a denaturing buffer according to Laemmli [18]. Electrophoretic separation was carried out at 23 mA per gel, 7 h, using calf thymus histones, which were run in parallel, in the same gel, as standards. After electrophoresis, the proteins were fixed and stained with Coomassie brilliant blue, and the ^{32}P labeled histones were detected by autoradiography of the slab gels using Kodak X-OMAT R films.

3. RESULTS

The changes in the in vitro ADP-ribosylation of nuclear proteins of rat liver, resulting from PB treatment of the animals, were studied by determining the specific radioactivity in the total protein precipitates as well as in the purified H1 and core histones after incubating the intact isolated nuclei in the presence of [^{32}P]NAD.

The 10% trichloroacetic acid precipitates analysed, contained the total ADP-ribosylated proteins from the nuclear suspensions free from [^{32}P]NAD precursor, non-covalently bound ADP-ribose oligomers and monomers which were further washed out by trichloroacetic acid and ethanol filtration [13,14].

Table 1 shows the incorporation of ^{32}P into the total trichloroacetic acid precipitates and purified histone fractions from control (C) and PB treated rat liver nuclei. Trichloroacetic acid precipitation

Table 1

[^{32}P]ADP-ribose incorporation into nuclear proteins in livers from control and PB treated rats

Animals	Total nuclear proteins	Histones	
		H1	Core
Control (cpm/mg protein)	6.87×10^5	23.7×10^5	8.34×10^5
Phenobarbital treated (cpm/mg protein)	17.2×10^5	32.0×10^5	8.93×10^5
Percentage increases after PB treatment	251	135	108

tests have been performed in triplicate. Data in table 1 represent the average, the individual results differing 1.9% from the mean.

The incorporation into total nuclear protein reveals that the specific activity attained in the nuclei of PB treated animals is approx. 2.5-fold the value found in the untreated controls.

To identify the acceptors of the (ADP-ribose) moieties among the different histone fractions, and particularly the protein components which are responsible for the hyper-ADP-ribosylation which we have observed after PB treatment, we determined the specific radioactivity in histone H1 and in the core histones purified from in vitro labeled nuclei. The results shown in table 1 reveal that histone H1 is the best ADP-ribose acceptor. For the control animals the specific activity attained in the histone H1 fraction (2.4×10^6 cpm/mg protein) is approx. 3.5-fold the value found in the total proteins (0.69×10^6 cpm/mg protein). The hyper-ADP-ribosylation of histone H1 observed after PB treatment (1.4-fold) is inferior, however, to the increase in the total protein ADP-ribosylation (3.5-fold). It is also slightly less than the increase in ADP-ribosyltransferase activity previously described (2-fold) [7]. Total core histones (H3, H2B, H2A, H4) showed a specific activity (0.83×10^6 cpm/mg protein) which is slightly higher (1.2-fold) than the value found for the total nuclear proteins (0.69×10^6 cpm/mg protein) in control animals, this value being barely increased (0.89×10^6 cpm/mg protein) after PB treatment as shown in table 1. The purity of the H1 and the core histone preparations from C and from PB rat liver was verified by SDS-PAGE (fig.1).

We have further investigated the protein components which are acceptors of the ADP-ribose residues, during the hyper-ribosylation induced by PB, by autoradiography of the gels after electrophoretic separation of the histone components. Fig.2 shows the autoradiograph of histone H1 purified from rat liver nuclei, indicating that [^{32}P]ADP-ribosylation is increased after PB treatment, the densitometric scanning of the 10 μg spot revealing a 1.44-fold increase.

The autoradiograph of the purified core histones is shown in fig.3. The left panel of fig.3 shows the presence of the 4 different components, H3A, H2B, H3 and H4, of the core histone preparation, which are virtually identical in C and PB preparations. The autoradiograph shown in the right panel of fig.3 reveals that fraction H2B is the main acceptor of the [^{32}P]ADP-R residues among the core histones, the densitometric scanning of the 40 μg spot revealing a 1.45-fold increase in total radioactivity associated to this fraction, in PB samples.

The specific activity of the total core histones was shown to increase only 1.08-fold (table 1). However, only H2B is strongly labeled – the other 3 core histones being weakly labeled or unlabeled. As H2B increased 1.45-fold similarly to the H1 histone (1.4-fold), the overall specific activity of the core histones would be expected to increase by

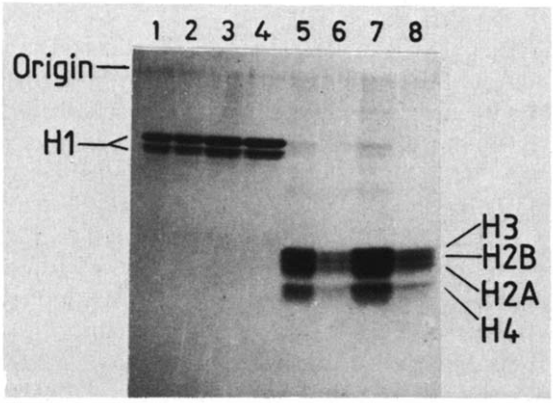

Fig.1. SDS-PAGE of purified histone fractions obtained from control (C) and phenobarbital (PB) rat liver nuclei, stained with Coomassie brilliant blue. Lanes: histone H1 of C, 1 and 3, and of PB, 2 and 4 (1.25 and 2.5 μg, respectively); core histone samples of C, 5 and 6, and of PB, 7 and 8 (5.0 and 2.5 μg, respectively).

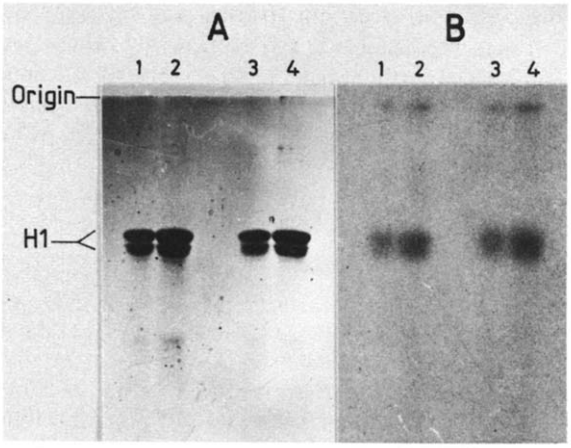

Fig.2. Incorporation of the label from [^{32}P]NAD into histone H1, in control (C) and in phenobarbital treated (PB) rat liver nuclei. (A) SDS-PAGE, stained with Coomassie brilliant blue. (B) Autoradiogram of the undried slab-gel developed after 46 h at −60°C showing ^{32}P incorporation into histone H1. Lanes: purified histone H1 samples of C, 1 and 2, and of PB, 3 and 4 (5 and 10 μg, respectively).

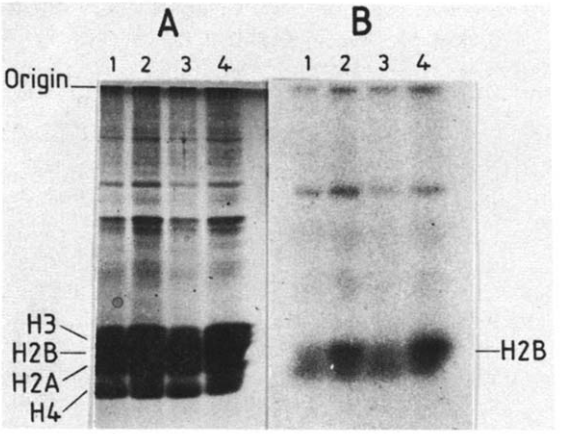

Fig.3. Incorporation of the label from [^{32}P]NAD into the core histones, in control (C) and in phenobarbital treated (PB) rat liver nuclei. Samples containing 20 and 40 μg proteins from C (lanes 1 and 2) and from PB (lanes 3 and 4) were electrophoresed, stained (panel A) and further autoradiographed (panel B) (see legend to fig.2).

only 25% of that (1.1-fold) which is very close to the value found (1.08-fold). No significant difference was found in histone H2A and H3 which appeared to be labeled to a lesser extent in the samples obtained from either C or PB treated animals. Taken together our results clearly show that histone H4 is not labeled in either C or PB treated animals and that no histone is strikingly labeled in a selective way by the increased enzyme activity.

In both cases, the presence of a weakly ^{32}P-labeled protein fraction can be observed in the autoradiographs which may correspond to an oligomeric aggregate of the histone fractions or to a non-histone protein contaminant.

4. DISCUSSION

The results reported here demonstrate that ADP-ribosylation of the rat liver nuclear proteins is markedly increased following administration of the xenobiotic, PB. The hyper-ADP-ribosylation observed 24 h after a single administration of PB, representing approx. 250% of the incorporation of the ADP-ribose moieties in the absence of treatment, is consistent with the induction of ADP-R-transferase activity previously described (200% at 24 h) [7].

The study of ADP-ribosylation of the purified histone fractions revealed that H1 is strongly ADP-ribosylated. Although hyper-ADP-ribosylation of this histone fraction was detected at the same stage of the response to the inducing agent, it showed a lower relative increase (140%) compared to the total trichloroacetic acid precipitated nuclear proteins.

Incorporation of [^{32}P]ADP-ribose into the core histones, measured in the purified fraction – consisting of H2A, H2B, H3 and H4 histone – showed that ADP-ribosylation is not significantly affected by PB treatment when the total fraction is considered. However, autoradiography performed after electrophoretic separation as well as calculations of the specific radioactivities demonstrate that among the core histones, H2B is the major acceptor of the ADP-ribose radicals, presenting an increase in the specific radioactivity after PB, comparable to that of histone H1.

The differences found both in the ADP-R-transferase activity [7] and related covalent modification of nuclear proteins in the intact nuclei, isolated from C and PB treated rat liver, preclude the existence of identical variations in vivo [8,19] although the hypothesis that a higher hyper-ribosylation of the histone fractions may occur in vivo cannot be ruled out. The relatively low

increases in the modification of these proteins observed in the nuclei, after incubation, could be due to previous hyper-ribosylation taking place in vivo during the 24 h period following PB administration which could limit the availability of free histone substrates.

Covalent modification of the histones, particularly the inter-nucleosomal histone H1, has been postulated to increase gene activity by improving the accessibility of specific DNA regions to RNA polymerase II [3,20,21]. The pleiotypic response induced by PB in the liver, characterized by a sharp increase in amino acid incorporation and partial change of the pattern of protein synthesis may be associated with a change in the supramolecular structure and function of particular genomic regions which could result from the increase in the ADP-ribosylation of histones.

The fact that the increase in the ADP-ribosylation of total nuclear proteins produced by PB in rat liver exceeds the increase observed in the histone fractions suggests that other nuclear proteins are covalently modified by the ADP-R-transferase in response to this gene modulating agent. Several non-histone proteins are known to undergo reversible ADP-ribosylation as a mechanism for metabolic regulation. This is the case for HMG [22] and for enzymes such as the Ca^{2+}, Mg^{2+} dependent endonuclease [23], RNA polymerase [24], ornithine decarboxylase [25], as well as the ADP-R-transferase itself, considered to be the best acceptor for the ADP-ribose radicals [26]. Studies in our laboratory have been undertaken to identify non-histone proteins which are ADP-ribosylated in rat liver nuclei, which could be involved in the molecular mechanisms of the adaptative response to PB.

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