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Impaired gene transcription and nuclear protein kinase C activation in the brain and liver of aged rats

Patrick J. Rogue, Marie-Françoise Ritz, Anant N. Malviya*

Laboratoire de Neurobiologie Moléculaire des Interactions Cellulaires (UPR 416 du CNRS), Centre de Neurochimie, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France

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The expression of the hsp70 and c-*fos* genes and the activation of nuclear protein kinase C (PKC) were studied in young and aged whole rats under heat-shock conditions. The induction of hsp70 and c-*fos* genes by heat shock were decreased several fold in the brain as well as in the liver of senescent animals. Nuclear run-off transcription assay indicated that this age-related impairment could be attributed to a block at the level of transcription. Nuclear PKC activation by heat shock was not apparent in old animals. Nuclear PKC involvement in the repression of transcription during senescence is postulated.

Aging; Heat shock; Nuclear PKC; Gene transcription

1. INTRODUCTION

Human diploid fibroblasts undergoing replicate senescence have become a widely accepted model for the study of the aging process [1]. Replicative senescence is associated with the transcriptional repression of various genes [2]. The rapid induction of the heat-shock protein (HSP) gene is one of the most characteristic biochemical response to heat shock (HS). Liu et al. [3] have found that aging human fibroblasts show a marked decrease in the magnitude of the induction of the hsp70 gene by HS. Similar results were found in rat fibroblasts [4] and in *Drosophila melanogaster* [5]. Furthermore, serum fails to induce c-*fos* transcription in late-passage senescent cells [6]. This is associated with an age-dependent decrease in AP-1 activity as well as altered AP-1 composition. Such a pattern of gene repression is selective, as other genes are normally induced in senescent cells [2,6].

The mechanisms underlying this transcriptional repression are not known. Protein kinases play an important role and could be involved in the regulation of transcription [7]. Protein kinase C, particularly nuclear PKC [8], has a function in the regulation of gene expression. Moreover, under heat stress, both cytosolic [9,10] and nuclear [11] PKC are stimulated, presumably due to increased diacylglycerol generation [11,12]. HS increased the activity of cytosolic PKC in rat fibroblasts [9]. PKC is also involved in the regulation of c-*fos* expression [13]. Furthermore, abnormalities of PKC have been described in Alzheimer's disease, which can be considered a pathological form of aging [14].

*Corresponding author. Fax: (33) 88 61 29 08.

The aim of the present study was to analyze the induction of gene expression and the activation of nuclear PKC during senescence. This study was performed in the whole animal which is more relevant to aging than fibroblasts [2]. Thermal stress was used as a stimulus. Indeed, HS not only induces the expression of hsp genes but also stimulates the transcription of c-*fos* [13]. Our results show that the activation of hsp70 and c-*fos* genes by HS are reduced several fold in the senescent animal, both in the brain and in the liver. These data prompt us to suggest that the decrease in nuclear PKC activation is involved in the repression of gene transcription in aging.

2. MATERIALS AND METHODS

Hybond N^+, [γ-^{32}P]ATP, [^{32}P]CTP, and [α-^{32}P]dCTP were obtained from Amersham. Phosphatidylserine, ATP and histone (type III-S) were from Sigma. DEAE-52 was from Servacel. Plasmid pH2.3, a genomic subclone of the human, hsp70 gene, was a generous gift from Dr. R. Morimoto, c-*fos* cDNA was generously donated by Dr. G. Labourdette.

2.1. *Conditions for animal treatments*

Male Wistar rats, either 2–3 months or 24 months of age, were heat stressed by placing them in a positive forced air incubator, initially maintained at 44°C, until the rectal temperature reached 42°C ± 0.5 (30–45 min). The incubator was then adjusted to 42°C and the animals were maintained at this temperature for an additional 50–55 min as shown by rectal temperature. Animals were monitored at regular intervals for signs of excessive discomfort. Following HS they were put back into a cage at room temperature and the brain and liver were removed at the indicated time.

2.2. *RNA isolation and analysis*

Total liver or brain RNA was prepared by the guanidium–isothiocyanate method, separated by electrophoresis on a 1% agarose-formaldehyde gel (5–10 μg RNA per lane), and transferred by capillary

action onto a Nylon membrane (Hybond N^+). The blots were prehybridized for 3 h at 42°C in a solution containing 50% formamide, 5 × SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.7), 5 × Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrolidone), and 0.5% sodium dodecyl sulfate (SDS). A probe, labeled by random priming with [^{32}P]dCTP (>3,000 Ci/mmol), was added to the prehybridization mix and hybridization was carried out for 48 h at 42°C. Stringent post-hybridization washes of the membranes were performed, followed by autoradiography. Levels of hsp70, c-*fos*, and β-actin mRNA were determined using specific cDNA probes (2.3 kb, 1.6 kb and 1.7 kb, respectively). The amount of labeled probe hybridized to specific mRNA bands was determined by quantitative densitometry of the autoradiograms using a Biocom RAG200 image analyzer.

2.3. *Nuclear run-off transcription assay*

Nuclear run-off transcription was performed as described [15]. Briefly, 5 × 10^6 nuclei, isolated as described below, were incubated for 1 h in the presence of 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 150 mM KCl, 2 mM dithiothreitol, and 0.5 mM each of ATP, UTP, GTP and 200 μCi of [α-^{32}P]CTP (3,000 Ci/mmol) in a total reaction volume of 500 μl. The reaction was terminated by chilling, and DNase and proteinase K were added. ^{32}P-Labelled RNA was phenol extracted and precipitated with trichloroacetic acid. Aliquots of the RNA samples containing an identical amount of radioactivity (10^7 cpm) were used for hybridization against hsp70, c-*fos* or β-actin probes immobilized on Hybond N^+ filters. Hybridization was carried out in a dot-blot apparatus (Bio-Rad) at 65°C for 48 h. Stringent post-hybridization washes of the membranes were performed, followed by autoradiography.

2.4. *Purification of rat liver nuclei*

Nuclei were isolated as described [8]. Small pieces of rat liver were homogenized in 8 vols. of a medium containing 1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 6.8. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 15 min at 1,000 × *g*. The pellet was suspended in the homogenization medium and mixed with a medium consisting of 2.4 M sucrose, 1.0 mM $MgCl_2$, and 10 mM phosphate buffer, pH 6.8, so as to give a final sucrose concentration of 2.2 M, and then centrifuged for 1 h at 100,000 × *g*. The resulting nuclear pellet was suspended carefully in 0.25 mM sucrose, 0.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5 (buffer A) and centrifuged for 10 min at 1,000 × *g*. The final pellet was resuspended in buffer A.

2.5. *Single step purification of nuclear PKC and PKC assay*

Purified nuclei were resuspended in a medium containing 2 mM EDTA, 0.5% Triton X-100 and 20 mM Tris-HCl, pH 7.5, sonicated in 5 ml aliquots six times for 10 s each with a 1 min interval between two sonications, incubated for 20 min on ice and centrifuged for 30 min at 100,000 × *g*. The resulting supernatant served as the source for nuclear protein kinase C and was loaded on a DEAE-cellulose column pre-equilibrated with a medium containing 20 mM HEPES, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1.0 mM dithiothreitol and 10% glycerol (buffer B). Subsequently, the DEAE-cellulose column was washed with buffer B supplemented with 30 mM NaCl. Finally, the enzyme was eluted from the column with buffer B supplemented with 120 mM NaCl. The fractions containing protein kinase C activity were pooled. Enzymatic assay was performed on the pooled fraction. The standard assay medium contained 20 mM Tris-HCl, pH 7.5, 1.2 mM EGTA, 5.0 mM $MgCl_2$, 3.5 mM $CaCl_2$, 16.6 μg phosphatidylserine, 20.0 μg lysine-rich histone (type III-S-Sigma), 2.0 mM phenylmethylsulfonyl fluoride and 20 μM (3–5 × 10^5 cpm) ATP. The incubation was done at 30°C for 15 min. Protein kinase C activity was determined by subtracting the activity measured in the absence of phosphatidylserine from the activity observed in the presence of calcium plus phosphatidylserine. Protein was determined according to the method of Bradford. Statistical significance was assessed using the Mann–Whitney *U*-test.

3. RESULTS AND DISCUSSION

The maximum induction of hsp70 gene expression in the liver was significantly decreased in aged rats as compared to the young animals (Fig. 1). Likewise c-*fos* expression due to heat shock (HS) was also diminished in aged animals (Fig. 2). Both hsp70 gene (Fig. 3) and c-*fos* expression (data not shown) were impaired in the brain of old rats. β-Actin mRNA levels in the liver as well as in the brain, used as an internal control, remained unaffected by HS. Whether the hsp70 and c-*fos* mRNAs decrease were due to a transcriptional event was determined by nuclear run-off assay. HS enhanced the expression of hsp70 and c-*fos* genes in the liver of young rats (Fig. 4). These results confirmed the decrease in transcription of both these genes with aging. Thus the age-dependent attenuation in the expression of these genes can be attributed to a transcriptional mechanism. The decrease in the hsp70 gene expression in senescent fibroblasts has been documented earlier [3]. We have demonstrated in this study that it also occurs in the whole animals both in nervous and non-nervous tissues. Furthermore, we also document that age-related impaired gene transcription also involves c-*fos* (Figs. 2 and 4).

In order to elucidate the underlying mechanism of this age-dependent transcriptional repression, we have examined the activation of nuclear PKC due to heat stress in the liver of young and old rats. Indeed since PKC is implicated both in the regulation of gene tran-

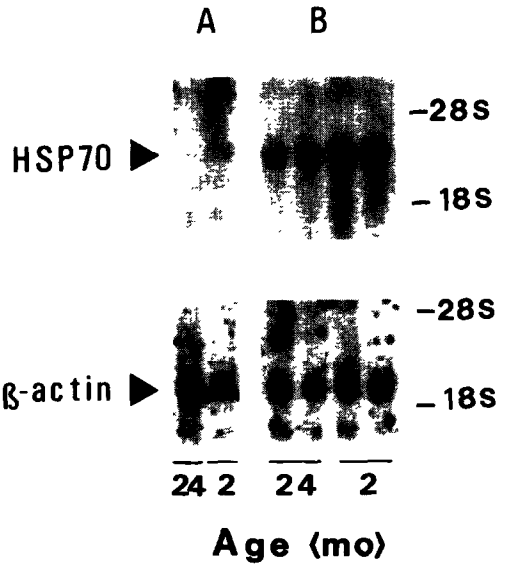

Fig. 1. Effect of HS on hsp70 (upper panel) and β-actin (lower panel) mRNA content in the liver of aged (24 months) and young (2 months) rats under control (A) and HS (B). Rats were decapitated 2 h after HS. Northern analysis was carried out as described in section 2. The probes for the hsp70 and β-actin genes recognized a major 2.5 kb and 2.2 kb band, respectively, in agreement with published data [16]. Maximum hsp70 induction by HS was an average 4.6-fold lower in aged animals compared to young animals.

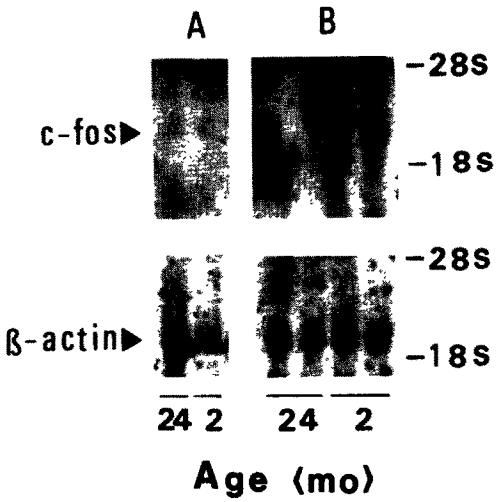

Fig. 2. Experimental conditions were the same as described in the legend to Fig. 1 except that the blots were hybridized with probes specific for c-*fos* (2.4 kb, upper panel) and *β*-actin (lower panel) mRNAs. Stimulation of c-*fos* expression in the liver by HS was an average 2.1-fold higher in young animals compared to aged rats.

scription [18] and in the HS response [11], we report here that the basal activity (i.e. without HS) of nuclear PKC (Table I) was significantly increased in the old as compared to the young animal. An analogous increase in the basal levels of other proteins have been described during senescence [19]. We also show here that the stimulation of nuclear PKC activity was significantly decreased in the aged rats subjected to the HS (Table I).

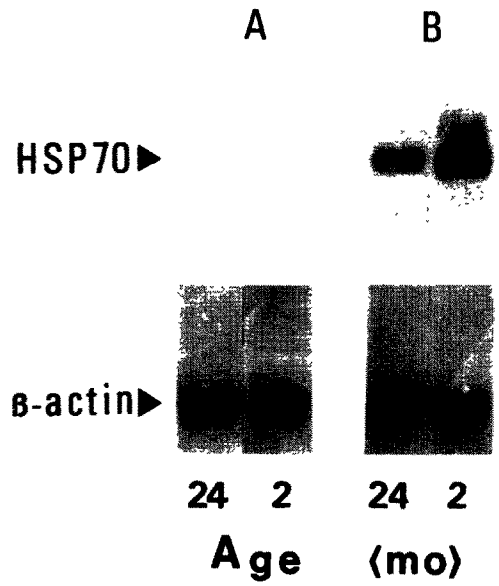

Fig. 3. Effect of HS on hsp70 (upper panel) and *β*-actin (lower panel) mRNA content in the brain of aged (24 months) and young (2 months) rats under control (A) and HS (B) conditions. In the brain, maximum induction of hsp70 expression occurred 1 h after return to the room temperature. The delays for hsp70 induction in the brain and in the liver are compatible with published data [17]. The maximum induction of the hsp70 gene in the brain is significantly decreased in aged animals as compared to the young animals, with an average 3.5-fold reaction. Similarly, stimulation of c-*fos* expression by HS was an average 2.7-fold higher in young animals as compared to old ones (data not shown).

Fig. 4. Nuclear run-off transcription of the hsp70 and c-*fos* genes. Nuclei were isolated from the liver of aged (24 months) and young (2 months) heat-shocked rats and nuclear run-off was performed as described in section 2.

How do age-dependent transcriptional repression and impairment of nuclear PKC stimulation relate? A logical mechanism would be phosphorylation of transcription factor such as HSTF by PKC. Unfortunately, this has not been demonstrated, although HSTF-1 and -2 possess a PKC consensus motif [20,21]. It has been recently shown that nuclear PKC*β* phosphorylates the transcriptional factor, C/EBP [22]. The promoter of the hsp70 gene is controlled by the CCAAT-box-binding protein (CBF), which is distinct from C/EBP [23]. Since CBF stimulates the hsp70 promoter one needs to understand if CBF is regulated by PKC. Similarities between the hsp70 promoter and c-*fos* promoter have been described [24]. Furthermore, PKC has been shown to be involved in the regulation of the expression of c-*fos* [13]. It is tempting to speculate that the age-dependent decrease in PKC activity leads to poorer activation of transcription through lack of phosphorylation of these factors. However, such a straightforward mechanism is unlikely. Indeed, phosphorylation of C/EBP by PKC leads to a decrease in the DNA binding [22]. Similarly, c-*fos* can function either as a transactivator of

Table I

Nuclear protein kinase C activation by heat shock

Age	2 months	24 months
Control	140.6	217.4^{+}
Heat shock	371.1*	230.1^{+}

Nuclear PKC*β* was partially purified from the liver of young (2 months) or old (24 months) control or heat-stressed rats as indicated in section 2. The figures are the specific activity in pmol ^{32}P incorporated/min/mg protein. Values represent the means of three independent experiments with replicates varying less than 10%.
*$P < 0.05$, comparison between HS and controls (*U*-test);
$^{+}P < 0.05$, comparison between young and aged animals (*U*-test).

transrepressor, and the influence of PKC phosphorylation at this level has been observed in a recent report [25]. An indirect mechanism may operate, possibly involving a protein phosphatase [26] or the product of tumor suppressor genes, such as Rb [27].

In conclusion, we have shown in the whole animal that senescence is associated with transcriptional repression of hsp70 and c-*fos* genes as well as impaired nuclear PKC activation.

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