BRIEF METHOD

A Sample-Saving Preparation to Extract DNA-Binding Proteins from Cardiac and Vascular Tissues

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The analysis of the changes in the DNA-binding activity of specific transcription factors is one of the approaches to study the ability of a tissue to develop and/or to adapt to external stimuli. Therefore, the availability of pure and functional nuclei, together with nuclear subfractions, is of crucial importance. A large number of methods have already been developed; however, they are commonly related to cultured cells or soft mammalian tissues. When applied to tough tissues, such as heart and vessels, they result in lower yield, so that large amounts of sample are always needed (Allfrey et al, 1964; Dignam et al, 1983; Fleischer and Kervina, 1974). The aims of our work were: (a) to prepare good quality nuclei and nuclear proteins from very small samples (30 to 50 mg) of muscle tissues (heart and vessels) by optimizing existent methods, in terms of homogenization and purification conditions; and (b) to verify the suitability of our preparation to study transcription factor activities, in particular nuclear factor- κ B (NF- κ B). We have specifically analyzed NF- κ B activation because it has recently gained interest in the cardiovascular field (Bourcier et al, 1997; Purcell et al, 2001) and because it was crucial to have a pure nuclear fraction avoiding any cytoplasmic contamination. In fact, NF- κ B is present in the cytoplasm, anchored to an inhibitory protein: I κ B α . After activation, I κ B α is degraded and NF- κ B gains access to nuclei for transcriptional regulation (Henkel et al, 1993; Lenardo and Baltimore, 1989).

To evaluate NF- κ B under quiescent (negative control) or activated (positive control) conditions, we have studied two groups of rats: a control group ($n = 3$) and

a lipopolysaccharide (LPS)-treated group ($n = 4$) (Shames et al, 1998).

Hearts and aortas were immediately washed with cold saline plus a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 25 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 μ g/ml pepstatin A), injected into the aortic root. A portion (30 to 50 mg) of cardiac or aortic tissue was finely minced, transferred into an all-glass tissue grinder with a clearance of 0.09 to 0.16 mm and volume of 2 ml (Wheaton), and homogenized in a 6 $\times$ volume of homogenization medium containing 0.25 M sucrose, 10 mM Tris (pH 7.5), and protease inhibitors. In the case of cardiac tissue, 0.1% of Nonidet P-40 was added to the medium. Homogenization was performed with two cycles of 10 strokes with 10 minutes "rest" in between.

The homogenate was centrifuged at 1000 $\times g$ for 10 minutes at 4° C. The supernatant was centrifuged at 100,000 $\times g$ for 60 minutes at 4° C to obtain the cytosolic fraction. The pellet was gently resuspended in 700 to 800 μ l of 0.25 M sucrose, 10 mM Tris, and 1 mM MgCl₂ (pH 7.5) and purified on a sucrose cushion (2.0 M, 4 ml) by centrifugation at 70,000 $\times g$ for 70 minutes at 4° C.

Nuclear pellets were lysed on ice, in a buffer containing 10 mM Tris (pH 7.5), 1 mM MgCl₂, 0.3 M NaCl, 25% glycerol, and freshly added 50 mM dithiothreitol, by vortexing (6 cycles/hour). The nuclear protein fractions were collected after centrifugation at 40,000 $\times g$ for 30 minutes at 4° C; they were then aliquoted, snap-frozen in liquid nitrogen, and stored at -80° C.

The integrity and purity of the nuclei were confirmed by fluorescence microscopic analysis, after staining with 4',6-diamidino-2-phenylindole and sulforhodamine 101, which stain DNA and proteic matrix, respectively. The nuclei appeared unbroken and almost completely free from cytoplasmic contaminants (data not shown).

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To detect NF- κ B activation, we have studied the cellular fractions by the immunoblotting technique. In detail, we have analyzed the presence of p65 (one of the two proteins that commonly constitutes NF- κ B) in the nucleus and the presence of I κ B α in the cytosol. To this end, proteins of both nuclear (30 μ g for heart or 8 μ g for aorta) and cytosolic (30 μ g for heart or 20 μ g for aorta) extracts were fractionated onto 10% (p65) or 12% (I κ B α) SDS-PAGE electrophoresis and transferred on polyvinylidene difluoride membranes. After blocking (5% nonfat dry milk), the membranes were immunoblotted with anti-p65 and anti-I κ B α antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Immune complexes were detected by anti-IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and analyzed by the enhanced chemiluminescence assay (ECL+plus; Amersham International, Arlington Heights, Illinois). The membranes were then rehybridated for octamer-1 and glyceraldehyde-3-phospho-dehydrogenase expressions to perform loading controls of nuclear and cytosolic extracts, respectively.

The results obtained by immunoblotting detection show that, under quiescent conditions (control group), p65 was almost absent in the nuclear fractions and I κ B α was present in the cytosolic compartment from both cardiac and aortic tissues (Fig. 1). As expected, when NF- κ B was activated (LPS-treated group), p65 markedly accumulated in the nuclear fractions and I κ B α levels were reduced in the cytosolic compartment from both tissues (Fig. 1).

Finally, we have analyzed NF- κ B DNA-binding activity in nuclear extracts by ElectroMobility Gel Shift Assay, using a 32 P-radiolabeled double-stranded oligonucleotide probe containing NF- κ B consensus binding site 5'-GGATCCTCAACAGAGGGGACTT-TCCGAGGCCA-3'. Samples (15 μ g of nuclear pro-

teins for cardiac and 2 μ g for aortic extracts) were incubated in a binding mixture [10 mM Tris, pH 7.8, 5% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 μ g of poly (dl-dC)] and 0.1 to 0.5 ng of labeled probe. The samples were loaded onto a 5% polyacrylamide gel in 1 $\times$ Tris-borate-EDTA. Gels were run at 4 $^{\circ}$ C for 2 hours at 20 mA, dried, and autoradiographed at -70 $^{\circ}$ C with Kodak MR film. For competition experiments, a 100-fold excess of specific unlabeled probe was added. Supershift assays were performed by incubating the nuclear extracts with 10 μ g of an anti-p65 antibody (Santa Cruz Biotechnology). To normalize the loading, we have analyzed DNA-binding activity versus octamer-1, an ubiquitous transcription factor whose site is present in many housekeeping genes, by using the following probe: 5'-GATCGAA-TGCAAATCACTAGCT-3'.

The results obtained with ElectroMobility Gel Shift Assay support those obtained by Western blotting analysis. No DNA-binding activity was detected in control hearts and aortas, whereas specific signals were observed in both tissues from LPS-treated animals. Signal specificity was validated by supershift and competition assays (Fig. 2). As expected, the DNA-binding activities of octamer-1 were constant both in control and LPS-treated tissues (Fig. 2).

Our results demonstrate that our sample-saving preparation does not produce any artifacts because it does not activate NF- κ B per se in samples from the control group (Figs. 1 and 2). It allows the identification of NF- κ B activation in samples from the LPS-treated group as demonstrated by: (a) accumulation of p65 in the nucleus and concomitant reduction of I κ B α levels

Figure 1.

Nuclear factor- κ B (NF- κ B) activation was evaluated as nuclear p65 and cytosolic I κ B α myocardial and aortic contents in cellular extracts from control and LPS-treated groups. A representative radiogram is shown. Octamer-1 and glyceraldehyde-3-phospho-dehydrogenase (GAPDH) expressions were analyzed as loading controls for the nuclear and the cytosolic extracts, respectively.

Figure 2.

NF- κ B binding activity was evaluated by ElectroMobility Gel Shift Assay in myocardial and aortic nuclear extracts from control and LPS-treated groups. A representative autoradiogram is shown. Competition: a 100-fold excess of specific unlabeled double-stranded probe was added. Supershift assays: 10 μ g of an anti-p65 antibody was added to the nuclear extracts. Octamer-1 binding activity was analyzed as loading control.

in the cytosol (Fig. 1); and (b) induction of NF- κ B DNA-binding activity in the nucleus (Fig. 2). Moreover, our preparation was useful to detect the expression and the activity of DNA-binding proteins other than NF- κ B, as shown by our findings on octamer-1 (Fig. 2).

Our preparation offers important advantages: (a) It is easy to perform and not time consuming. (b) It avoids pooling samples. (c) It offers the possibility of studying a regional physiologic/pathologic process that involves only a portion of the organ of interest. (d) It can be used on human biopsy specimens, thus overcoming the limitations of a morphologic study.

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