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Translocation of HSP27 and MKBP in Ischemic Heart

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ABSTRACT. **HSP27 and MKBP translocate from the cytosolic to myofibril fraction in ischemic rat heart as demonstrated by immunoblotting. Immunohistochemistry analysis showed that ischemia enhances the Z line labeling of HSP27 and MKBP. Two dimensional gel electrophoresis showed that ischemia increases the hyperphosphorylated form of HSP27. These data suggest that HSP27 and MKBP may be involved in the Z line protection against postischemic reperfusion injury.**

Key words: HSP27/MKBP/translocation/ischemia/myofibril/oxidative stress

The small heat shock protein family is comprised of heat shock protein 27 (HSP27), myotonic dystrophy protein kinase-binding protein (MKBP), αA,B-crystallin and p20 (1–3). HSP27 is induced by heat shock, heavy metals, serum, hormones, cytokines or H_2O_2 (2, 3) or by brain ischemia-reperfusion (4). HSP27 locates predominantly in the cytosol, but it becomes associated with the nucleus or actin-filaments in response to various forms of stress (1–3).

Recently, we found that MKBP enhances myotonic dystrophy protein kinase activity and protects it against heat inactivation (5). Additionally, we reported that MKBP and HSP27 are upregulated in the neonatal myocardium and they lie at the Z lines, intercalated discs and the nuclei (6). Although it was reported that ischemia induces αB-crystallin translocation in the heart (7), the translocation of HSP27 or MKBP in ischemic tissues is unknown. This is the first report on the translocation of HSP27 and MKBP in ischemic tissue.

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Abbreviations: HSP27, heat shock protein 27; MKBP, myotonic dystrophy protein kinase-binding protein; MAP kinase, mitogen activated protein kinase.

Materials and Methods

Materials

Anti-HSP27 antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), ECL Western blotting detection kit from Amersham (Buckinghamshire, England), Vectastain ABC kit from Vector Laboratories (Burlingame, CA, USA). Anti-MKBP antibody was raised against the GST-MKBP fusion protein (5).

Perfusion procedure

The protocol of the animal experiments was approved by the Committee of the Ethics of Animal Experiments of Yamaguchi University School of Medicine. As previously reported (8–10, 15), the hearts of male Wistar rats weighing about 200 grams were perfused with Krebs-Hensleit solution gassed with 95% O_2-5% CO_2 at a constant pressure for about 10 min for stabilization and elimination of blood contamination, and then subjected to global ischemia for 10–40 min (some hearts were reperfused). The control hearts were perfused with the oxygenated buffer for 10 min. There was no difference in the localization of HSP27 and MKBP in the heart perfused with the oxygenated buffer for 10 min and that perfused for 50 min (data not shown). After perfusion, the ventricles were quickly frozen in liquid nitrogen and stored at −70°C.

Subcellular fractionation

As previously reported (8–10), the frozen hearts were homogenized in STE buffer containing 320 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 50 mM NaF and 0.2 mM phenylmethanesulfonyl fluoride, 20 μM leupeptin and 0.15 μM pepstatin A. The homogenates were centrifuged at 1,000 × g for 10 min, and the supernatant at 100,000 × g for 60 min. The 1,000 × g pellet, 100,000 × g pellet, and the 100,000 × g supernatant were designated as P1, P2, and S, respectively. Protein concentrations were determined by the method of Lowry *et al.* (16).

Immunoblotting and quantification

The samples were subjected to SDS-polyacrylamide gel (12.5%) electrophoresis by the method of Laemmli (17) and then immunoblotted according to Towbin *et al.* (18) with modifications (8–10, 15). The blots were blocked with skim milk, incubated with either the 2,000-fold diluted anti-HSP27 or the anti-MKBP antibody for one hr at room temperature, and the proteins were visualized by an ECL Western blotting detection kit. The amounts of proteins on the immunoblots were measured by an image analyzer (Densitograph AE-6900, Atto, Tokyo, Japan) (8–10, 15) and expressed in arbitrary units (100 units for the mean value of the zero-time sample of the S fraction and that of 40 min ischemia sample of the P1 fraction).

Two-dimensional (2-D) gel electrophoresis

The P1 and the S fractions of control and ischemic (20 min) hearts were subjected to 2-D gel electrophoresis by the method of O'Farrell (19) followed by immunoblotting with anti-HSP27 antibody. The spots were quantified by the image analyzer.

Immunohistochemistry

The ventricles were removed, immersed in OCT compounds, and rapidly frozen in liquid nitrogen. After sectioning, the specimens were fixed on glass slides with 50% acetone-50% methanol at −20°C for 10 min, washed with PBS and treated with 3% H_2O_2. After washing with PBS and blocking with 5% horse serum in PBS, the specimens were incubated with anti-HSP27 antibody diluted 100-fold in 1% BSA in PBS for one hr at room temperature and immunostained by the avidin-biotin-peroxidase complex (ABC) method of Hsu *et al.* (20). The details were as described in our previous reports (10, 15).

Results

Translocation of HSP27 and MKBP to the myofibril fraction during ischemia

The P1, P2 and S fractions represent the nucleus-myofibril, membrane and cytosolic fractions, respectively, as we showed previously (8–10). Figure 1 shows that a large portion of both HSP27 and MKBP are distributed to the P1 and S fractions but not to the P2 fraction, and that ischemia induces translocation of HSP27

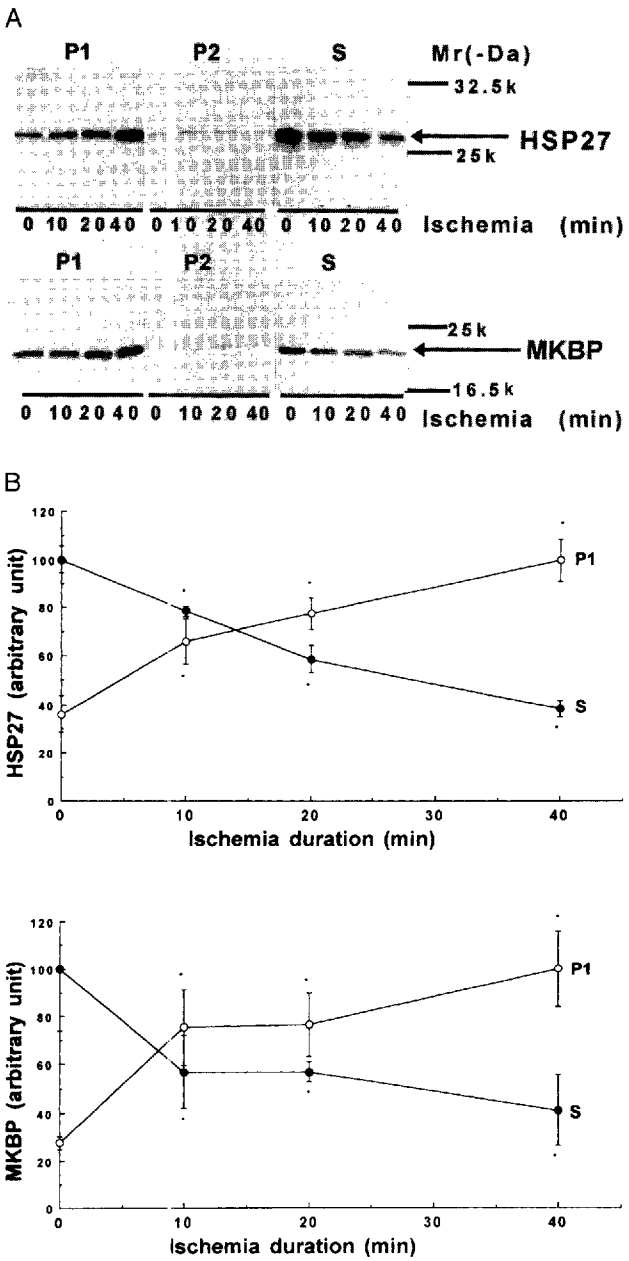

Fig. 1. Translocation of HSP27 and MKBP from the S to P1 fraction during ischemia. Panel A shows the representative immunoblots and panel B shows the amount (arbitrary units) in the P1 fraction (open circles) and the S fraction (closed circles) quantified from the immunoblots of four independent experiments (*: $P<0.05$ vs. 0 time). The same amount of protein (P1, P2: 20 μg, S: 10 μg) was applied for each fraction. For details, see text.

and MKBP from the S to P1 fraction. These data were supported by immunohistochemical observations as shown in Figure 2. As we reported previously (5, 6), HSP27 is localized at the Z lines and nucleus (Fig. 2A), while MKBP is at the Z lines and intercalated discs in the control heart (Fig. 2B). After 40 min of ischemia, the staining of the striations at the Z lines were enhanced for both HSP27 (Fig. 2C) and MKBP (Fig. 2D). Together with the biochemical findings (Fig. 1), these data showed that ischemia causes translocation of HSP27 and MKBP from the cytosol to myofibril Z lines. Additionally, HSP27 also appeared to translocate to the nucleus after ischemia (Fig. 2C).

Two-dimensional (2-D) gel analysis of HSP27 phosphorylation

We examined the phosphorylation of HSP27 by 2-D gel electrophoresis-immunoblotting using anti-HSP27 antibody as shown in Figure 3 and Table I. From basic to acidic isoelectrophoretic points, there were three spots (a, b and c) that are supposed to correspond to HSP27 with phosphorylation of no, one and two residues, respectively (13). In contrast with the finding on quiescent cultured cells in which most of HSP27 exists in a non-phosphorylated state (spot a) (2), our observations showed that about 43% of HSP27 is phosphorylated at one residue (spot b) in the P1 and S fractions in

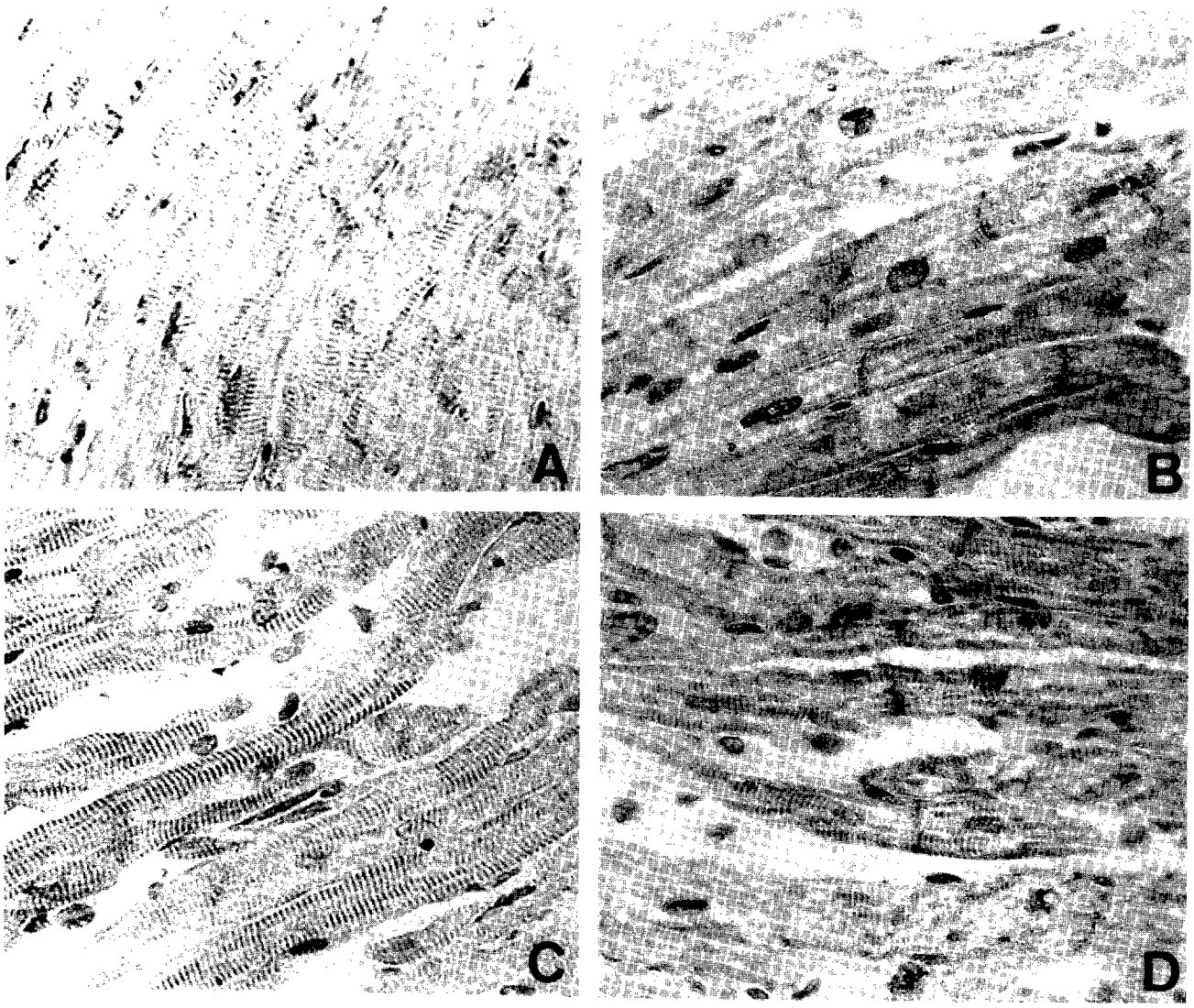

Fig. 2. Immunohistochemical demonstration of enhanced staining of HSP27 and MKBP at the Z lines in ischemic heart. The control heart (A, B) and ischemic heart (40 min; C, D) are labeled with anti-HSP27 (A, C) or anti-MKBP antibody (B, D). The staining of the striations (Z lines) of HSP27 and MKBP and nuclear staining of HSP27 were enhanced after ischemia. For details, see text.

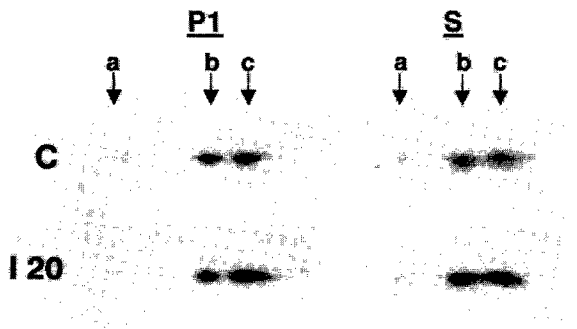

Fig. 3. Two-dimensional (2-D) gel electrophoresis-immunoblotting of HSP27 in the P1 and S fractions of the control and ischemic (20 min) heart. The P1 (20 μg) and S (10 μg) fractions were subjected to 2-D gel electrophoresis and immunoblotting using anti-HSP27 antibody. The spots a, b, and c are thought to show no, one, and two phosphate incorporated forms of HSP27, respectively. Ischemia induced the transition of HSP27 to hyperphosphorylated state, though the extent is small. The quantification is shown in the Table. A representative of two similar experiments is shown.

the control heart (Fig. 1). Ischemia increased the ratio of HSP27 in spot c/spot b in the P1 and S fractions, showing the transition to a hyperphosphorylated state after ischemia. However, the small amount of the nonphosphorylated form and the small extent of the transition to the hyperphosphorylated form appeared to be unassociated with the large extent of the HSP27 translocation. Under the same conditions, we could detect neither plural spots for MKBP nor the change in its phosphorylation state in ischemia (data not shown). The inhibitors for protein kinase C (chelerythrine) or p38 MAP kinase (SB 203580) had no effect on the myofibril translocation of HSP27 and MKBP in the ischemic heart or that of HSP27 in the myogenic H9c2 cells rendered ischemic by metabolic inhibition, though the p38 MAP kinase inhibitor, SB 203580, was toxic to the perfused heart (data not shown). As we reported that p44/p42 MAP kinase is not activated by ischemia but by reperfusion (8), this kinase cannot be involved in the translocation of HSP27 or MKBP in ischemic heart. Thus, a novel mechanism besides protein kinase-mediated phosphorylation may be causing the translocation.

Table I. DISTRIBUTION OF HSP27 SPOTS ON 2-D GEL

Spots		a	b	c	c/b
Estimated Phosphate		0	1	2	ratio
P1	Control	0.2	43.3	56.5	1.3
P1	Ischemia	N.D.	31.7	68.3	2.2
S	Control	6.1	43.7	50.2	1.1
S	Ischemia	2.1	38.1	59.8	1.6

The amounts of HSP27 spots were quantified from the immunoblots of 2-D gels shown in Figure 3 and expressed as the ratio (%) to the sum of the three spots in the fraction.

Discussion

This study is the first to demonstrate the translocation of small heat shock proteins, HSP27 and MKBP, from the cytosol to the myofibril Z lines in the ischemic heart (Figs. 1, 2). Ischemia also appeared to induce the nuclear translocation of HSP27.

We also showed that ischemia increased HSP27 phosphorylation (Fig. 3), but the change in phosphorylation state appeared to be unassociated with the translocation. Additionally, although p38 MAP kinase and PKC are activated in ischemic heart and each can phosphorylate HSP27 *in vitro* (10–14, 21), inhibitors for these kinases did not inhibit the translocation of HSP27 or MKBP in the heart or the myogenic cells under ischemic state. In support of our interpretation, it was reported that nonphosphorylatable HSP27 in a mutant cell line translocates to the nucleus in the same manner as phosphorylatable HSP27 does in a wild type cell line (22).

It was reported that ischemia induces αB-crystallin translocation to the Z line in the heart (1, 7). What then is the physiological significance of the translocation of HSP27 and MKBP in ischemic heart? It was reported that overexpression of HSP27 conferred resistance to actin fragmentation in the cells after oxidative stress of H_2O_2 (22). Recently, the same group reported that H_2O_2 at the same concentrations induces fragmentation of F-actin in fibroblasts with low HSP27 expression, but it reorganizes F-actin and activates p38 MAPK/MAPKAP 2 in the vascular endothelial cells with high HSP27 expression (11). On the basis of these data, they proposed that the p38 MAPK/HSP27 pathway plays a central role in the microfilament response to oxidative stresses (11). In addition, Martin *et al.* reported that the overexpression of αB-crystallin and HSP27 protects cardiomyocyte against ischemic injury (23). Moreover, we reported that MKBP and HSP27 are upregulated in the neonatal heart (6), possibly as an adaptive response to the oxidative stress induced by hypoxia (during labor) and reoxygenation (after birth).

Taken together, it is speculated that ischemia induces translocation of HSP27 and MKBP to the Z lines, where the proteins protect actin filaments in the Z lines against oxidative stress generated during postischemic reperfusion.

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