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# ANGIOTENSIN RECEPTOR BLOCKER CANDESARTAN BINDS TO AND UPREGULATES MOLECULAR CHAPERONE HSP90 IN THE HIPPOCAMPAL CA1 NEURONS

## —— A POSSIBLE MECHANISM OF NEUROPROTECTION BY ANGIOTENSIN RECEPTOR BLOCKER ——

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**Abstract**
Antihypertensive angiotensin II type 1 receptor blockers (ARBs) have neuroprotective effects that were independent of the reduction of blood pressure. We have previously shown that vulnerable hippocampal CA1 neurons underwent delayed cell death through mitochondria–dependent apoptotic pathway after global cerebral ischemia, but ARB protected these neurons from cell death.
To elucidate the mechanism of neuroprotection by ARB candesartan, candesartan–specific binding proteins were first investigated using candesartan–affinity column and rat hippocampal tissue. And, the affinity of ARB and the binding protein was further examined by BIAcore binding assay. Then, the distribution and expression of the protein were characterized by immunohisotochemistry and Western blots.
By affinity column study, molecular chaperone heat shock protein 90 (HSP90) was identified as a binding target of candesartan. BIAcore assay proved functional binding of candesartan to HSP90. Immunohistochemistry showed increased HSP90 expression in the hippocampal CA1 neurons as early as 1 day after candesartan treatment. Westen blots confirmed temporal changes in HSP90 expression.
HSP90 maintains the stability of the proteins as a molecular chaperone and has been recently proven to inhibit apoptosis in in vitro studies. Our data suggest that HSP90 may play an important role in neuronal protection by ARB treatment through blocking mitochondria–dependent apoptosis.

**Key words** : angiotensin II type 1 receptor, heat shock protein 90, molecular chaperone, apoptosis, hippocampus, ischemic injury

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## Introduction

The humoral renin-angiotensin system (RAS) regulates blood pressure and electrolyte/fluid homeostasis through effects on vascular smooth muscle, the adrenal gland, and kidney. Angiotensin II (AT II) is the main functional peptide in the RAS which binds to two specific G-protein-coupled receptors, AT II type 1 and 2 receptors (AT1R and AT2R)[1)]. AT1R mediates vasoconstriction, angiogenesis, and inflammation, whereas AT2R mediates vasodilatation, apoptosis, and inhibition of proliferation[1, 2)]. Angiotensin II type 1 receptor blockers (ARBs) are used worldwide for the treatment of hypertension, and clinical trials have revealed protective effects against stroke that were independent of the reduction in blood pressure[3-5)]. However, the mechanism of protective effects on the brain has been obscure.

Experimental studies also showed amelioration of ischemic injury after focal cerebral ischemia in animals treated with ARB[6-8)]. And, we have recently shown that pretreatment with ARB candesartan reduced ischemic injury in the hippocampal CA1 neurons after global cerebral ischemia in rats[9)]. The present study was conducted to clarify the mechanism for the protection of these neurons against ischemic damage. As a first step of the study, candesartan-specific binding proteins were investigated using candesartan-affinity column and rat hippocampal tissue. And, candesartan was found to bind to heat shock protein 90 (HSP90) besides its primary target AT1R. The binding affinity of candesartan to HSP90 was further examined by BIAcore binding assay. Subsequently, temporal and spatial expression of HSP90 in the hippocampus after candesartan treatment was examined by immunohisotochemistry and Western blots, and possible role of HSP90 in ARB-mediated neuronal protection is discussed.

HSPs are also called molecular chaperons and maintain the stability of the proteins by preventing misfolding and aggregation of polypeptides[10,11)]. HSP90 is constitutively expressed in the cytosol and mediates the ATP-dependent refolding of heat-denatured proteins[12-14)]. HSP90 is thought to play important roles in the conformational maturation of nuclear hormone receptors and in the stress response of the cells[14,15)]. Recent investigations have shown that, in addition to the role in quality control of the proteins, HSP90 can regulate cell death by direct interaction with the factors in the apoptotic pathway in vitro[14)]. Since neuronal death in rodent cerebral ischemia models is at least partly caused by apoptotic pathway[16,17)], the role of HSP90 as an inhibitor in the pathway is expected.

## Materials and Methods

### Candesartan-affinity Column Chromatography

Candesartan-sepharose was prepared using candesartan (Takeda Pharmaceuticals, Tokyo, Japan) and epoxy-activated sepharose 6B (GE Health care, Buckinghamshire, UK). Rat brain whole cell fraction in 10 mM Tris-HCl (pH 7.4) was applied to the column quilibrated with the same buffer and washed with 20 column volumes of the buffer containing 0.15 M NaCl. After washing the column extensively, binding proteins were eluted with 0.14 mM Candesartan in the same buffer. The eluants were analyzed on SDS/7%PAGE[18)] or by immunoblotting[19)] using an polyclonal anti-bovine HSP90 antibody. After electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue R-250 in a mixture of 25% isopropyl alcohol and 10% acetic acid ; they were destained with 10% isopropyl alcohol and 10% acetic acid or by immunoblotting using an anti-HSP90 antibody and alkaline phosphatase anti-rabbit IgG antibody (Sigma-Aldrich Japan, Tokyo, Japan)[20)]. In this study, we used Epoxy-activated Sepharose 6B as a control.

### Real time analysis of HSP90-candesartan interaction by surface plasmon resonance

The BIAcore appratus (BIAcore 2000, Uppsala, Sweden) was used to examine a direct interaction between HSP90 and candesartan. The procedures were described in detail in the previous reports[21)]. Candesartan was covalently linked to the surface of a flow cell on a sensor chip using amine-coupling kit. The binding kinetics of HSP90 to immobilized candesartan was determined by increasing HSP90 (1.15-9.2 $\mu$M) in running buffer at a flow rate of 10 $\mu$M/ml. The kinetic parameters of the binding reactions were determined using BIAevaluation version 3.0 software. The dissociation rate constant ($k_d$)

was determined from a plot of $\ln(R_0/R)$ *versus* time, with $R$ being the surface plasmon resonance signal at time $t$ ; the association rate constant ($k_a$) was determined from a plot of $\ln(\mathrm{abs}(dR/dt))$ *versus* time. The apparent equilibrium dissociation constant was calculated from the kinetic constant $K_D = k_d/k_a$.

**Candesartan administration**

Male Sprague-Dawley rats (weight 250-300 g) were given daily oral administration of 1 mg/kg body weight of candesartan (Takeda Pharmaceuticals, Tokyo, Japan) dissolved in 2 mL/kg of saline or only saline for 7 consecutive days (days 1 to 7). The rats were sacrificed on day 1, 3 and 7 for immunohistochemistry and Western blot. The dose of 1 mg/kg/day was determined from the maximum neuroprotective effects observed in the previous study[9]. Experimental protocols were approved by Akita University School of Medicine animal experiment committee.

**Immunohistochemistry**

Anesthetized animals were perfused with 10 U/mL heparin in saline and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed for 24 hours in the same fixative. The brains were coronally sectioned on a vibratome at a 50-$\mu$m thickness. Free-floating sections were immunohistochemically processed to label AT1R and HSP90. Briefly, the sections were incubated in 20% normal goat serum (NGS) in PBS with 0.3% Triton-X and exposed to the rabbit polyclonal anti-human AT1R (1 : 250 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal anti-bovine HSP90 (1 : 250, described above) for 2 hours at room temperature. The sections were subsequently exposed to Texas red-conjugated secondary antibody (1 : 1,000 ; Alexa 546, Invitrogen, Carlsbad, CA, USA) for AT1R labeling or to biotinylated anti-rabbit IgG followed by fluorescein avidin antibody (1 : 200 ; Vector Laboratories, Burlingame, CA, USA) for HSP90 staining. The sections were embedded with DAPI-containing medium for nuclear counterstaining and examined under confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany).

**Western blot**

Approximately 50 mg of samples from the hippocampus was excised and processed with homogenizer Physcotron NS-310E (Nichion, Tokyo, Japan) in 10 mM Tris-HCl (pH 7.4). Proteins were separated by SDS-PAGE on a 7% Tris-glycine gel and transferred to a polyvinylidene difluoride membrane (Invitrogen). The membrane was incubated in the primary antibodies against AT1R (1 : 1,000, described above) or HSP90 (1 : 1,000, described above) for 2 hours at 37°C and then incubated in the appropriate phosphatase-conjugated secondary antibody. For quantitative study, the stained membranes were scanned and densitometry was performed with ImageJ softeware. To confirm a consistent protein loading for each lane, membranes were also stained for $\beta$-actin.

**Statistical analyses**

All data were expressed as mean ± standard deviation (SD). The statistical differences among the groups were analyzed by a 1-way ANOVA and Dunnetts's test for multiple comparisons. Significance was accepted with $p<0.05$.

## Results

Affinity-column chromatography detected candesartan-binding proteins in the soluble fraction of the hippocampal homogenates (Fig. 1A). The major bands were approximately at 90 kDa and 40 kDa of molecular weight. Some other proteins were also detected as faint bands. Further analyses using Western blot identified the proteins of major bands as HSP90 and AT1R, respectively (Fig. 1B). These results suggest that HSP90 and AT1R are direct binding targets of the ARB candesartan.

Direct interaction between candesartan and HSP90 was characterized by BIAcore binding assay (Fig. 2). Utilizing BIAcore evaluation software, the kinetic parameters $k_d$, $k_a$ and $K_D$ were determined. The binding curves were fitted to a simple bimolecular binding algorithm with $x^2=0.332$. The results of the surface plasmon resonance analysis with immobilized candesartan and HSP90 were $k_a=7.36\times10^3$, and $k_d=5.51\times10^{-3}$. The apparent equilibrium dissociation constant ($K_D$) was $7.48\times10^{-7}$ M.

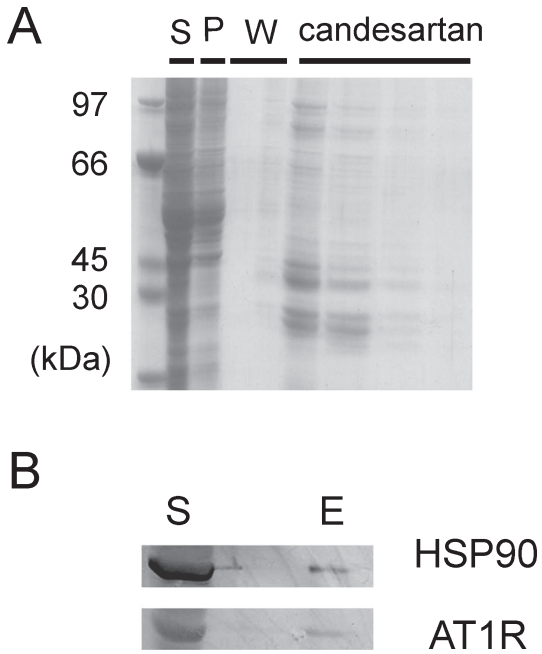


Fig. 1. Candesartan affinity-column chromatography. Rat hippocampal whole cell fraction in 10 mM Tris-HCl (pH 7.4) were applied to candesartan-affinity column, washed with 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, and proteins were eluted with 10 mM candesartan. The eluants with 0.14 mM candesartan were analyzed on SDS/7% PAGE, followed by staining with Coomassie Brilliant Blue (A), and immunoblotting using an anti-AT1R antibody or an anti-HSP90 antibody (B). In panel A, *S*, *P*, and *W* denote applied samples, pass-through fraction, and washed proteins, respectively. In panels B, *S* and *E* denote applied samples and eluted proteins, respectively.

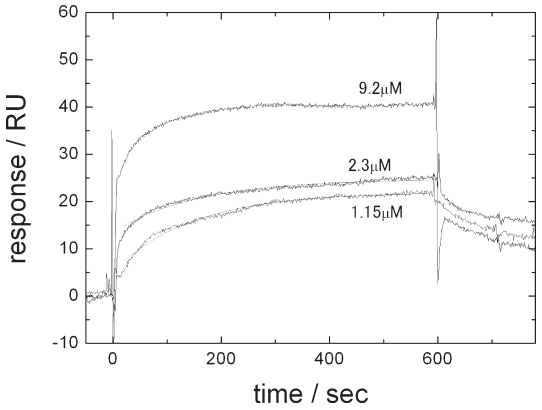


Fig. 2. Surface plasmon resonance analysis of the interaction between HSP90 and candesartan. A sensorygram for the binding of HSP90 to candesartan is shown. Different concentrations of HSP90 at 1.15, 2.3 and 9.2 $\mu$M were tested. RU : resonance units.

Immunohistochemical labeling of AT1R showed diffuse punctate staining along the vascular lumen (Fig. 3A-C), consistent with the previous observations. However, virtually no positive structures were observed in the pyramidal neurons in the hippocampus (Fig. 3D-F). No specific staining was detected in the specimens processed without primary antibody against AT1R (data not shown).

HSP90 immunohisochemistry showed that the protein was constitutively expressed in the hippocampal CA1 neurons in normal rats (Fig. 4A-C). HSP90 staining showed faint, diffuse cytosolic pattern and consisted of fine punctate labeling. The staining was not evident in non-neuronal cell populations in the hippocampus. The intensity of HSP90 immunolabeling was obviously higher at 1 day after candesartan administration than in normal rats (Fig. 4D-F). But, no substantial change in staining was observed in vehicle treated animals (data not shown).

Western blot analyses also showed an increase in HSP90 at 1 and 3 days in the hippocampal tissue after candesartan treatment compared to normal animals, whereas no increase was observed in vehicle treated animals (Fig. 5A, B). Quantitative OD analyses confirmed a significant increase in HSP90 at 1 and 3 days in candesartan-treated group, however, no changes were detected in vehicle-treated group (Fig. 5C, $n=4$ in each group).

## Discussion

ARBs are clinically used as antihypertensives and the treatment of hypertension reduces the risk of initial and recurrent stroke. In addition to the effects of blood pressure reduction, large clinical trials of ARBs, such as the Study on Cognition and Prognosis in the Elderly (SCOPE)[4] and the Losartan Intervention For Endpoint reduction in hypertensive study (LIFE)[3] suggested blood pressure-independent protective effects on the brain. More recently, ARB treatment in acute[5] and chronic[22] stage after stroke has been reported to improve progno-

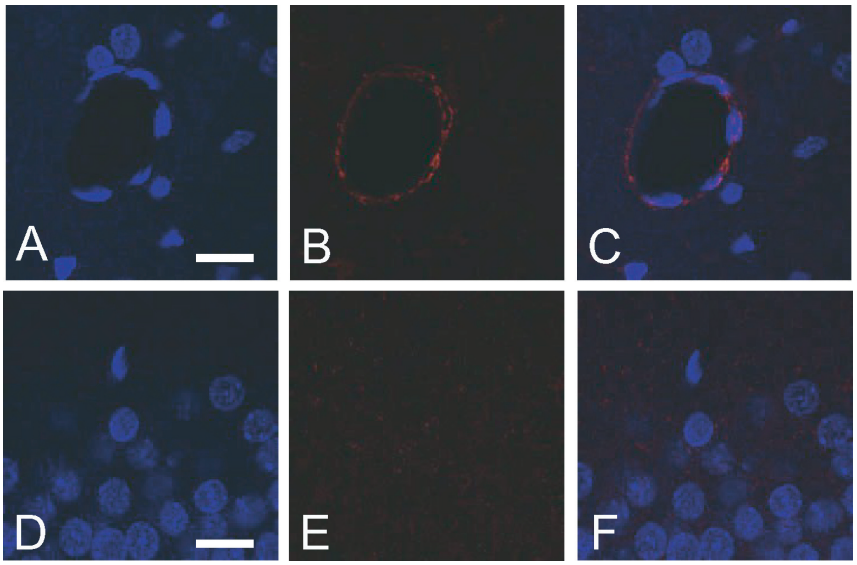


Fig. 3. Immunohistochemical labeling of angiotensin II type 1 receptor (AT1R) in the normal hippocampal CA1 subregion. Fine immunopositive dots were diffusely distributed in the vascular endothelium (A–C). However, virtually no positive structures in the pyramidal neurons (D–F). A, D : DAPI, B, E : HSP90, C, F : merged. Scale bar=20 $\mu$m.

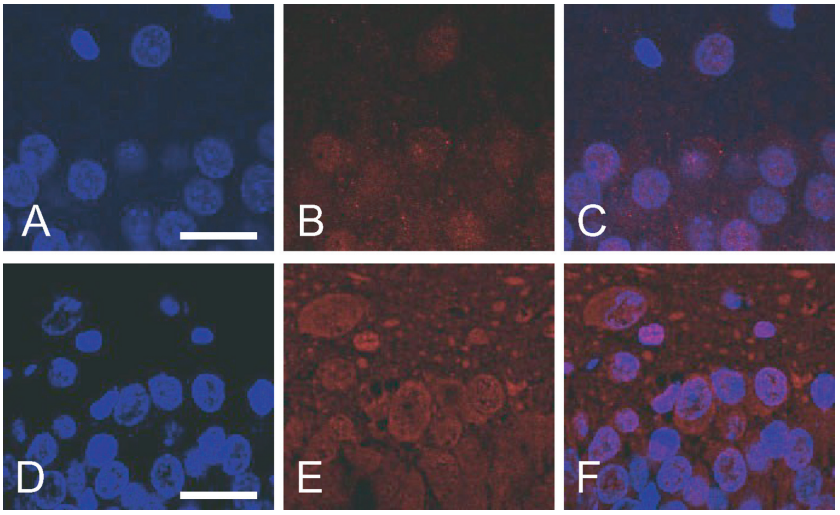


Fig. 4. HSP90 immunostaining in the hippocampus. Faint punctate staining was evenly distributed in the cytosol of the hippocampal CA1 pyramidal neurons in normal rats (A–C). The staining became more intense one day after candesartan administration (D–F). A, D : DAPI, B, E : HSP90, C, F : merged. Scale bar=20 $\mu$m.

sis. Candesartan treatment in acute stage reduced the risk of cardiovascular events and mortality compared to placebo treatment after 1-year follow up[5]. Another ARB eprosartan reduced cerebrovascular events by 25% compared to calcium channel blocker nitrendipine when the treatments were started in the chronic stage[22]. These

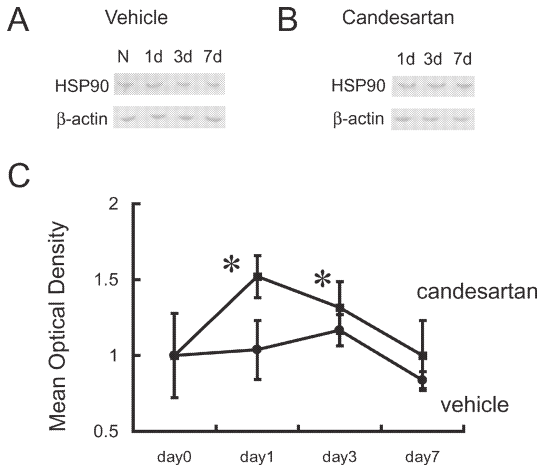


Fig. 5. Western blot of HSP90 in the hippocampus after candesartan administration. HSP90 appeared as a single band at the molecular weight 90 kDa. The density of the band increased at 1 day after candesartan administration (A). The density was not obviously changed in vehicle treated animals (B). Consistent protein loading per lane was confirmed by similar density of the bands of b-actin (A, B). Densitometry (C) showed a significant increase at 1 and 3 days in candesartan group (*$p<0.01$, $n=4$ at each time point). N : normal, 1d : 1 day, 3d : 3 day, 7d : 7day. Error bars are SD.

clinical data suggest the protective effects of ARB on the brain beyond the reduction in blood pressure.

Recent experimental studies also showed amelioration of ischemic cerebral damage after AT1R blockade. Intracerebroventricular injection of the ARB irbesartan did not reduce the blood pressure, but ameliorated neurological deficits after focal cerebral ischemia in normotensive rats[7]. Multiple intravenous injections of candesartan reduced infarct volume and improved neurological outcome without changing the blood pressure[8]. And the authors reported that pretreatment with candesartan dramatically reduced ischemic injury in the hippcampal CA1 neurons after global cerebral ischemia[9]. Several mechanisms of protection in ischemic neuronal tissues were proposed. Vascular NADPH oxidase has been recently identified as a source of superoxide[23] in one of the signaling pathways downstream of AT1R activation, where kinases and transcription factors are activated by complex signaling cascades[2,24]. AT1R blockade by candesartan reduced superoxide production and subsequent hippocampal CA1 injury after global cerebral ischemia[9]. This mechanism of protection was supported by the previous results that excessive reactive oxygen species (ROS) produced after ischemia induces activation of the mitochondria-dependent apoptosis pathway in the rodent model of cerebral ischemia[17,25]. However, the lack of the AT1R in the neurons is opposed to the previous hypothesis that ARB protects neurons by direct inhibition of NADPH oxidase through AT1R blockade. The present study is the first to show HSP90 upregulation in the neurons by ARB treatment and may explain the mechanism of its neuroprotection.

Accumulated evidence suggests that the delayed neuronal death after cerebral ischemia is at least in part, caused by apoptotic cell death presumably through mitochondrial pathway[17, 26, 27]. This pathway is initiated by release of cytochrome c from mitochondrial intermembrane space to the cytoplasm. Cytochrome c interacts with the CED homologue Apaf-1 and dATP, forming apoptosome to activate caspase-9. Active caspase-9 subsequently activates caspase-3 and caspase-2, -6, -8, -10[28, 29]. Caspase-3, one of the executor caspases, activates DNase and directly cleaves DNA repair enzyme poly(ADP-ribose) polymerase (PARP), leading cells to apoptosis[17, 26]. In the hippocampal CA1 neurons, global cerebral ischemia induces cytochrome c release immediately after the insult, then caspase-9 activation followed by caspase-3 activation and DNA fragmented apoptotic cell death[17]. HSP90 mRNA was shown to be induced in rat hippocampus after global cerebral ischemia[30] and HSP90 expressing cells were not injured by focal cerebral ischemia[31]. And recently, HSP90 has been found to inhibit the mitochondrial pathway of apoptosis by suppressing caspase activation in the downstream of cytochrome c-mediated oligomerization of Apaf-1 and activation of caspse-9 in *in vitro* studies[14]. These results suggest that HSP90 expression contributes to ischemia tolerance presumably through blocking mitochondria-dependent apoptosis.

In the present study, HSP90 was constitutively expressed in the CA1 neurons, and increased at one day after candesartan administration. Direct binding of candesartan to HSP90 was confirmed by affinity-column and BIAcore binding studies. There were bands of some other proteins in affinity-column study, however, BIAcore

assay showed the apparent equilibrium dissociation constant ($K_D$) of HSP90 and candesartan was $7.48 \times 10^{-7}$M, suggesting specific binding. These results strongly suggest the direct interaction between candesartan and HSP90. Transcription of HSPs is regulated by heat shock factor-1 (HSF-1), an activator of HSP genes[32]. Under normal condition, transcriptional activity of HSF-1 is repressed by serine phosphorylation[33]. Stresses or low HSP condition activates the DNA binding activity of HSF-1 and subsequently, HSF-1 enters nucleus to form trimer, resulting in transcription of HSPs[34]. Conversely, HSF-1 activity is negatively regulated in HSP rich condition[32, 35]. We suppose the change in HSP condition by binding between candesartan and HSP90 is a cue to induce HSF-1 activation and subsequent HSP90 upregulation. It is of great interest to investigate the HSF-1 trimerization and phosphorylation status after candesartan treatment.

Focusing on the hippocampal CA1 neurons and the ischemic insult, the authors previously demonstrated that global cerebral ischemia induced mitochondrial apoptosis in these vulnerable neurons[16, 17] and ARB candesartan protected these neurons from cell death[9]. The present study showed direct binding of candesartan to HSP90 and HSP90 upregulation in these hippocampal CA1 neurons. Since HSP90 can inhibit mitochondrial apoptosis by blocking the cytochrome c-mediated oligomerization of Apaf-1 and activation of caspase-9, protection of the CA1 neurons may be the result of suppressed apoptosis by HSP90 upregulation in these cells. The exact mechanisms of candesartan binding to HSP90 upregulation and relationship between HSP90 and neuroprotection need to be clarified, however, our data suggest the involvement of HSP90 in neuroprotection by ARBs.

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