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FAST TRACKS

Role of MAPK Phosphorylation in Cytoprotection by Pro-Vitamin C Against Oxidative Stress-Induced Injuries in Cultured Cardiomyoblasts and Perfused Rat Heart

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Abstract The reactive oxygen species (ROS) are known to be generated upon post-ischemic reperfusion (I/R) of the heart, and to injure cardiac muscle cells. The hydrogen peroxide-induced mortality of rat cardiomyoblasts H2c9 was markedly inhibited by previous administration with auto-oxidation-resistant pro-vitamin C, the 2-*O*-phosphorylated derivative (Asc2P) of ascorbic acid (Asc). The cytoprotection was partially counteracted by an inhibitor of MAPK (mitogen-activated protein kinase) kinase (MEK) as shown by DNA strand cleavage assay and mitochondrial dehydrogenase assay. Immunostains indicated that phosphorylated MAPK increased in the hydrogen peroxide-treated cardiomyoblasts, and that this action was moderately inhibited by Asc2P and restored nearly to the initial, pretreatment level by combined administration of the MEK inhibitor and Asc2P. The I/R-induced cell injuries in perfused rat hearts as estimated by extracellular release of the cardiac enzyme CPK were inhibited by 2-*O*-α-glucosylascorbic acid (Asc2G) and Asc, whereas the observed cytoprotection for the cardiomyoblasts was partially counteracted by the MEK inhibitor. The increase in phosphorylated MAPK in I/R-operated hearts was moderately inhibited by pro-vitamin C, but restored nearly to the normal non-operated level by combined administration with the MEK inhibitor. This is in contrast to no alteration in levels of non-phosphorylated MAPK for all the cases examined as shown by Western blots, consistent with results of immunostains for the cardiomyoblasts. The inhibitory effect of the MEK inhibitor on MAPK phosphorylation was, therefore, suggested to counteract the cytoprotective effects of pro-vitamin C via a thorough interruption of the phosphorylated MAPK signaling pathway. This was not true of ROS-related events; the scavenging effects of Asc2G and Asc on hydroxyl radicals generated from I/R-operated heart were not affected by combined administration with the MEK inhibitor, as shown by the spin-trapping DMPO-based ESR method. J. Cell. Biochem. 90: 219–226, 2003.

Key words: pro-vitamin C; ROS; MAP kinase

The water-soluble vitamin C (L-ascorbic acid, Asc) works as an anti-oxidant by reacting with oxidized vitamin C, dehydroascorbic acid, by reactive oxygen species (ROS) in vivo. Recently, the auto-oxidation-resistant pro-vitamin C, Asc-2-*O*-phosphate (Asc2P) and 2-*O*-alpha-D-glucosylated derivative (Asc2G), were shown to resist oxidized vitamin C [Eguchi et al., 2003a,b]. Intracellular Asc derived from pro-vitamin C was also shown to be retained within the cell longer than that from administered Asc [Tsukaguchi et al., 1999]. Asc2P is scarcely converted to vitamin C by alkaline phosphatase, and Asc is transported by Asc transporters into cells [Kanatate et al., 1995; Saitoh et al., 1997]. Likewise, Asc2G is scarcely converted to vitamin C by α-glucosidase, and Asc is transported by Asc transporters into cells [Saitoh et al., 1997; Kageyama et al., 1999].

Free radicals are serious threats to health, and in some cases result in I/R injury, producing oxygen-derived free radicals such as H_2O_2 and O_2^-, which are known to induce cell death.

The mechanism whereby cytotoxicity is not exhibited when treated by the Asc derivative remains to be analyzed in terms of free radical generation. The plasma lipoproteins exposed to

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aqueous peroxy radicals undergo no hydroperoxidation until depletion of endogenous Asc that scavenges ROS [Fujiwara et al., 1997]. The Asc is depleted more promptly than other plasma ROS scavengers such as SH groups, alpha-tocopherol, bilirubin, and urate.

The heart can recover from I/R injury when certain genes are expressed, such as *c-fos*, *c-jun*, and *Egr-1* [Mizukami et al., 1997]. These are rapidly up-regulated during post-I/R [Mizukami et al., 1997]. This finding indicates that upstream kinases for mitogen-activated protein kinase (MAPK) are important regulators of nuclear transcription activity [Eguchi et al., 2003b]. MAPK is a serin/threonine protein kinase whose activity is up-regulated by the phosphorylation of tyrosin and therionine residues catalyzed by MAPK/extra-cellular signal-regulated kinases (MEK) [Crews et al., 1992]. I/R injuries have been shown to act as upstream kinases for MAPK activation. The MAPK activation pathway is present in nuclei of ischemic hearts [Mizukami and Yoshida, 1997; Eguchi et al., 2003b].

In the present study, we show that provitamin C protected the cardiac muscle cell against H_2O_2-induced injury. Then, we analyzed the function of the activation of the MAP kinase family using cardiac muscle cells and hearts of rats.

MATERIALS AND METHODS

Cell Cultures

The embryonic rat heart-derived cell line H9c2 was obtained from ATCC (CRL 1446); the cells exhibited many of the properties of skeletal muscle such as formation of multinucleated myotubes due to fusion of myoblasts and response to stimulation by acetylcholine. H9c2 cells were cultured in DMEM (Nissui Pharm., Tokyo, Japan) supplemented with 10% dialyzed and inactivated fetal bovine serum (FBS).

H_2O_2 Treatment

H9c2 cells (2×10^4 cells) were seeded in a 24-well microplate OR 24-well microplates and were precultured for 22 h. The medium was then replaced by fresh DMEM-10% FBS containing Asc2P (100 μM) and incubated for 22 h. The cells were administered for 48 h with H_2O_2 (200 μM) and concurrently with Asc2P/derivative. MEK inhibitor PD98059 (New England Biolabs, Beverly, MA) dissolved in 0.1% DMSO was administered 30 min before H_2O_2 treatment. The control cells received DMEM-10% FBS containing no H_2O_2 agent or Asc2P/derivative.

Microtiter Photometric Assay for Mitochondrial Dehydrogenage Activity: WST-1 Assay

The treated or control cells underwent aspiration of medium and received 300 μl of phenol red-free DMEM-10% FBS and 30 μl of WST-1 solution (Wako, Tokyo, Japan). After 3 h incubation, the medium was transferred into a 96-well microplate and was measured with 450 nm (Bio-Rad, San Jose, CA microplate photometer).

Immunofluorescent Staining

H9c2 cells (2×10^4 cells) were seeded in a chamber slide. The cells were fixed with 4.5% paraformaldehyde solution for 15 min at room temperature. Following fixation, the cells were incubated for 60 min at 37°C with antibody phospho-MAPK (Tyr-204) (New England Biolab) at 1:100 dilution in PBS containing 1% bovine serum albumin. They were then washed with PBS and incubated for 40 min at 37°C with FITC-conjugated anti-rabbit IgG (Santa Cruz Pharmaceuticals, Santa Cruz, CA) at 1:100 dilution in PBS containing 1% bovine serum albumin. The apoptotic-like cells was detected by TUNEL method (TAKARA, Kyoto, Japan) The cells were viewed with a confocal microscope.

I/R-Induced Injury in the Heart

Male Wistar rats (weighing 300–350 g) were deprived of food for 18 h before the experiments but were allowed to drink water freely. Two hours before the Langendorff perfusion, Asc2G 1.92 mg/kg was dissolved in a physiological saline solution and administered by intravenous injection (i.v.); controls received an injection of physiological saline solution. The rats were anesthetized by intraperitoneal injection (i.p.) of sodium pentobarbital (25 mg/kg) and heparin (300 IU). The hearts were prepared for Langendorff mode as described previously [Eguchi et al., 2003b]. The buffer was maintained at 37°C and gassed with 95% N_2 and 5% CO_2 containing 50 μl Asc or MEK inhibitor U 0126 (Promega, WI).

After 15 min of ischemia, the hearts were reperfused with buffer gassed with 95% O_2 and 5% CO_2. The activity of acreatine phosphokinase (CPK) in the buffer was measured using

the CPK-HA test (Wako, Osaka, Japan) at indicated times after reperfusion.

Subcellular Fractionation

The frozen hearts were homogenized in 2 ml STE buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM $MgCl_2$, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupetin, 0.15 μM pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM glycerophosphate, and 2.5 mM sodium pyrophosphate) homogenizer (Nichion rika seisakusho, Tokyo, Japan; NS-310E) for 30 s at maximum speed. The samples were mixed with 2 vol STE buffer and centrifuged at 1,000*g* for 10 min at 4°C. The pellet containing the nuclear division was mixed with 1 ml TE buffer (20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, and 10 mM β-mercaptoethanol) containing 50 mM NaCl, 20 μM leupetin, 0.2 mM phenylmethanesulfonyl fluoride, and 0.15 μM pepstatin A, and mixed with 1% Triton X-100 for 60 min on ice, then centrifuged at 15,000*g* for 30 min at 4°C. The supernatant containing the cellular membrane was centrifuged at 100,000*g* for 60 min at 4°C.

Electrophoresis and [Western] Blots

The subcellular fractionation sample was electrophoresed in polyacrylamide gels in the presence of SDS and transferred to polyvinylidene difluroide membranes (0.45 μm, Millipore Co., Bedford, MA). It was then blocked with 5% non-fat dry milk (Yukijirushi, Tokyo, Japan) in tris-buffered saline containing 0.05% Tween-20 (Wako, Tokyo, Japan) and rat antibody. MAPK and *p*-MAPK (New England Biolab) were added, and the mixture was kept at room temperature for 45 min. Horseradish peroxidase and anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) were added to a secondary antibody at 4°C over night. The reactive bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

Measurement of Ascorbyl Radicals by ESR

ESR spectra were measured with a JEOL ESR apparatus type JES-FR30 and recorded in a Krebs–Henseleit buffer containing 100 mM DMPO. The X-band spectrometer was equipped with an ESPRIT computer system at 100 kHz magnetic field modulations. Instrument settings were as follows: magnetic field, 336,000 mT; microwave power, 4 mW; Gsweep width, 5 mT; modulation width, 0.125 mT; time constant, 1 s. ESR observations were made in a flat-quartz cell (Labotech, LLC04) at 25°C.

RESULTS

Cytoprotection by Asc2P Against H_2O_2-Induced Viability Reduction

The H9c2 cardiomyoblasts exposed to H_2O_2 underwent reduced viability as assessed by microtiter photometry for mitochondrial dehydrogenase activity (Fig. 1A). The administration of Asc2P prevented the reduction of H_2O_2-induced viability. Administrations of the MEK inhibitor PD098059 also prevented H_2O_2-induced viability reduction as extensively as did administration with Asc2P alone, and were advantageous at a dose of 10 μM over Asc2P alone in terms of cytoprotection.

The H_2O_2-treated H9c2 cells were confirmed by microscopy to be deformed and degenerated (Fig. 1B). In addition, extensive cleavages of cellular DNA strands typical of symptoms for apoptosis were histologically detected by the TUNEL method in the H_2O_2-treated H9c2 cells (Fig. 1D). Apoptosis-like cell death in H_2O_2-treated H9c2 cells was detected. In contrast, apoptosis-like cell death was not detected in H_2O_2-treated H9c2 cells administered with Asc2P.

Activity of MAPK

The phosphorylation of MAPK (P-MAPK) was increased in H9c2 cells treated with H_2O_2 at 200 μM and rat hearts treated with I/R. These H_2O_2-induced or I/R increases in P-MAPK were prevented by administration with Asc2P, while the MEK inhibitor treatment prevented H_2O_2-induced activity of P-MAPK (Figs. 1C and 2B).

We showed that P-MAPK was activated by I/R injury in rat hearts through the Langendorff mode and that P-MAPK was activated by H_2O_2 (data not shown). These results indicate that activity of P-MAPK protects against H_2O_2-induced cell death.

Effects of Asc in Perfusates of the Isolated Rat Heart on Extracellular Release of CPK in the Langendorff's Perfusion Apparatus

The release of CPK into the buffer prepared for Langendorff mode took place after

reperfusion. In the present study, the buffer in Langendorff mode containing 50 μM Asc indicated the extracellular Asc concentration, while the α-glucosidase that changed Asc2G to Asc was not present in the buffer. After I/R injury in the rat heart, release of CPK into the buffer decreased with the use of Asc2G (Fig. 2A). Interestingly, the release of CPK into the buffer increased with the use of MAPK-inhibitor and Asc2G. These data indicate that Asc2G

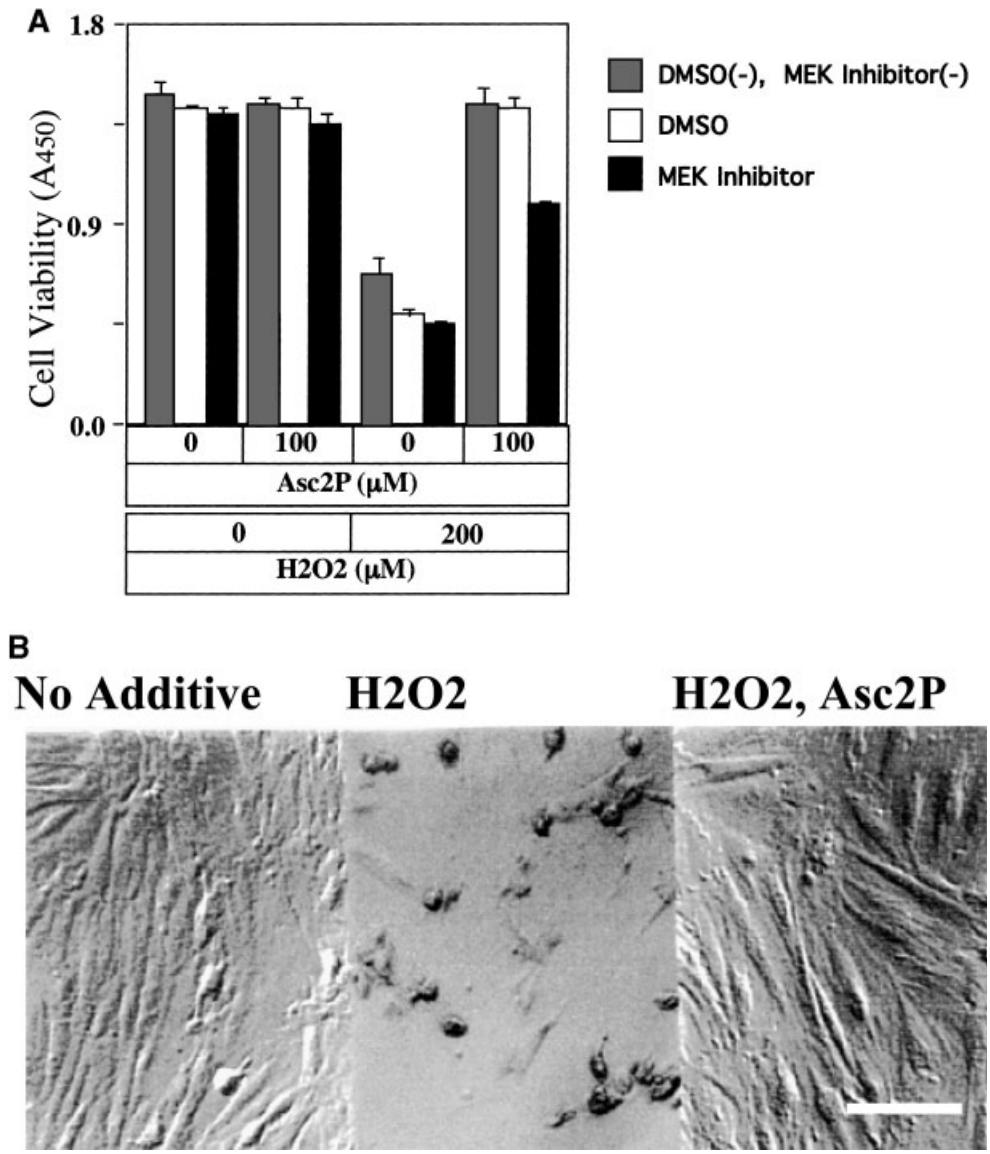
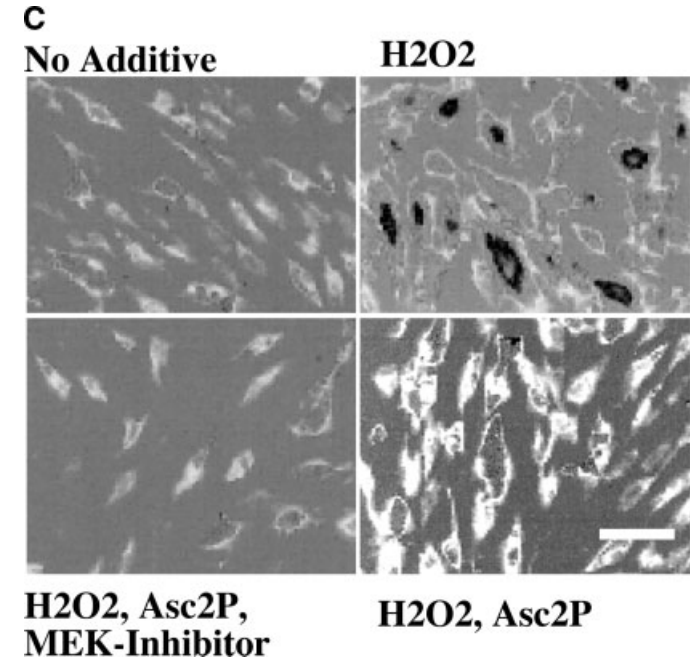


Fig. 1. Effects of pro-vitamin C against H_2O_2-induced injury in rat cardiomyoblastic cell line H9C2. **A**: The cytoprotective effects of pro-vitamin C ascorbic acid-2-*O*-phosphate (Asc2P) against H_2O_2-induced injury. The cells were administered with Asc2P and/or the MEK inhibitor PD98059, then treated with H_2O_2 for 48 h, followed by estimation of cell viability by the photometric assay using the formazan-forming dye WST-1. **B**: Microphotographs of H9c2 cells administered with H_2O_2 and/or Asc2P. The cells were previously administered with Asc2P or not, then treated with H_2O_2 for 22 h, followed by the phase-contrast microphotography. The scale indicates 50 μm. **C**: Effects of Asc2P and/or MEK inhibitor on phosphorylation of MAPK (P-MAPK) in H_2O_2-treated H9c2 cells. The cells were previously administered with Asc2P and/or the MEK inhibitor PD98059, and treated with H_2O_2 followed by immunostaining using anti-phosphorylated MAPK antibody and FITC-conjugated anti-IgG antibody. The scale indicates 50 μm. **D**: DNA strand cleavages in the vicinity of the capillary vessel of the I/R- or sham-operated heart of rats with or without Asc2G administration. The livers were excised and fixed at 150 min after reperfusion. They were sliced and stained by TUNEL method for the I/R-subjected or the unsubjected region that is similarly located in the middle lobe of the liver. The TUNEL stain was detected with a confocal laser scanning fluorescence microscope and processed so as to be expressed more densely in proportion to frequency of the DNA cleavages. Cells undergoing apoptosis-like death are indicated by arrows. The scale is 100 μm.



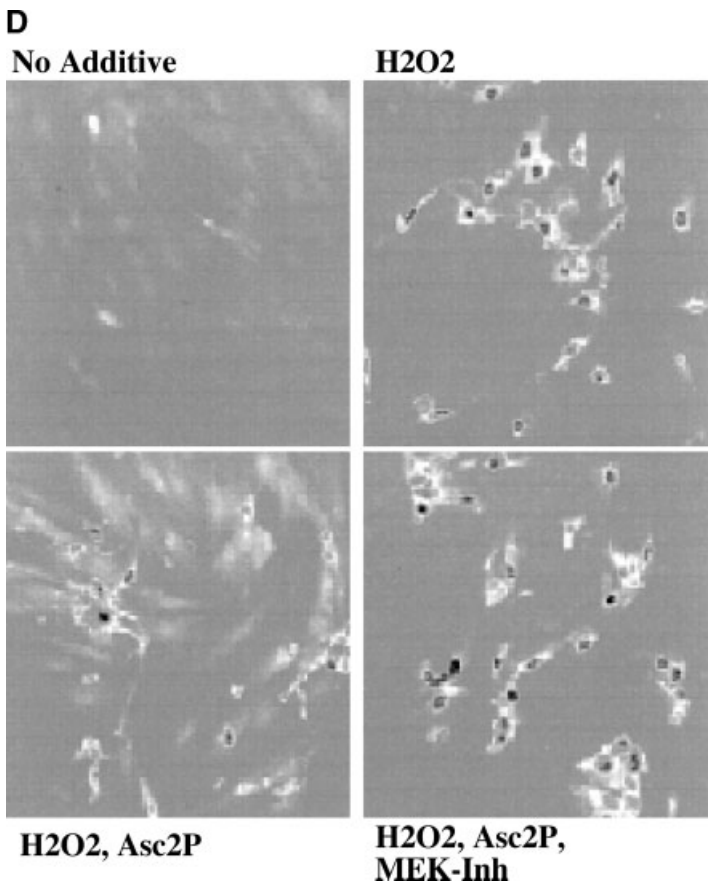


Fig. 1. (*Continued*)

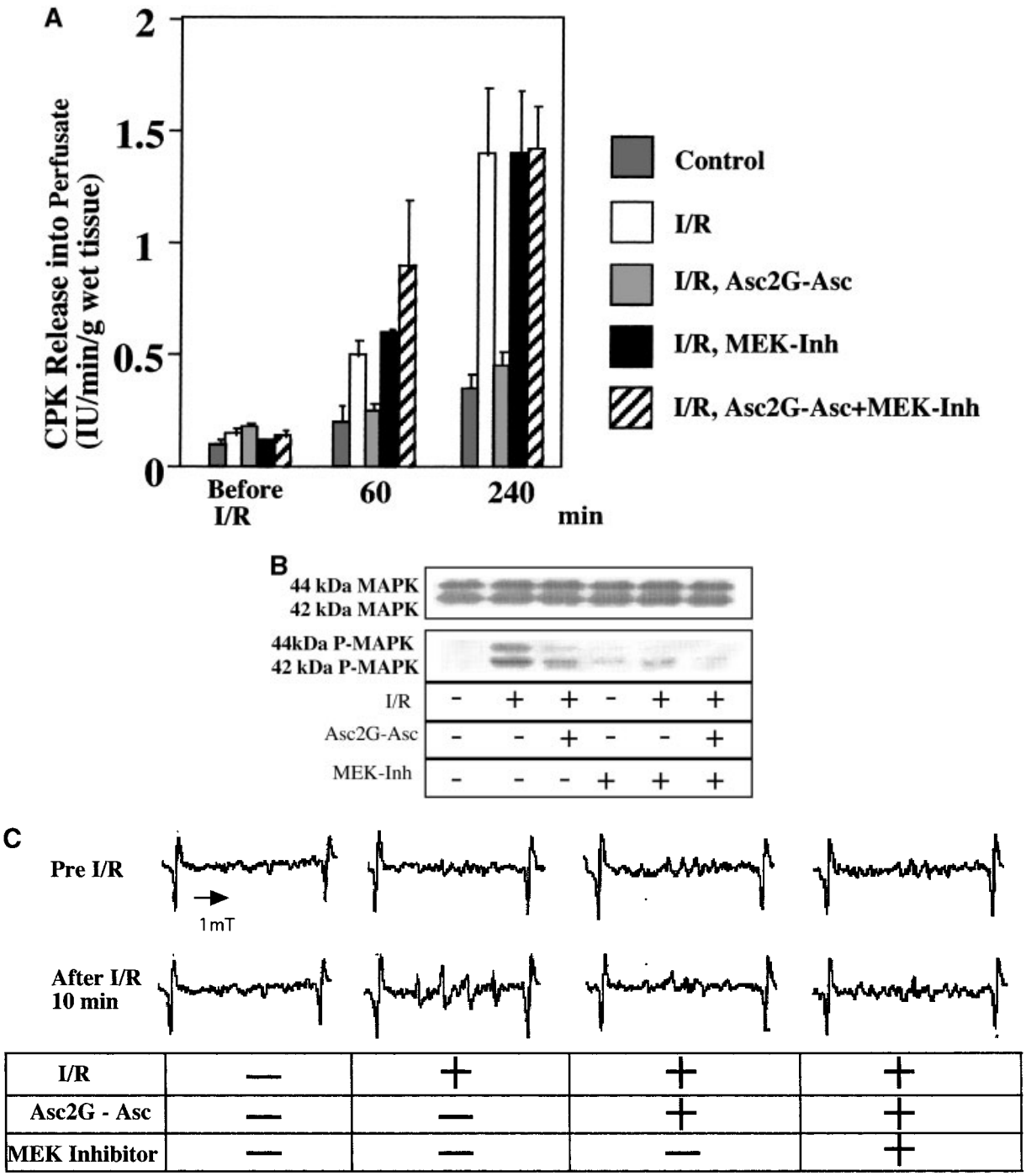


Fig. 2. Preventive effects of pro-vitamin C against I/R-induced cell injury in the perfused rat heart. **A**: Effects of ascorbic acid (Asc) in perfusates of the isolated rat heart on extracellular release of CPK from I/R-treated cardiomyoblasts. Rats underwent intravenous administration with or without Asc2G at a dose equivalent to 2 mg Asc/kg body weight. After 60 and 240 min, the heart was isolated and left in the perfusate of the Langendorff's perfusion apparatus. The heart was subjected to 15-min ischemia and subsequent reperfusion, and was immediately administered Asc at 50 μM and/or the MEK inhibitor U 0126 via the perfusate. The release of CPK from the heart into the perfusate was quantified as an indicator for cytolysis of the cardiomyocytes at graded times after I/R. The data shown were typical of three to six independent experiments for each treatment, respectively. The bar represents the SD of measurement points in triplicate in each experiment. **B**: Effects of Asc2G on alteration and P-MAPK in the isolated rat heart subjected to I/R. At graded times after outset of reperfusion, the nuclear fractions of the perfused heart were separated and assessed by Western blots. **C**: The ESR spectra of homogenates of the rat hearts treated in a manner similar to that described in (A). The heart homogenate received the spin-trapping agent DMPO and, after 1 min, was measured for ESR signals attributable to hydroxyl radicals. The peaks at both ends are attributed to the paramagnetic standard compound MnO, and are utilized as the basis for estimating the relative ESR intensity.

protected against I/R injury in the rat heart, and MAPK also need protection.

Hydrogen Peroxide Radicals in the Langendorff's Perfusion Apparatus

Hydrogen peroxide radicals in the Langendorff buffer spectrum appear as characteristic doublet signals (Fig. 2C). They were decreased in Asc2G-injected rats and Asc2G-MAPK inhibitor-injected rats. In the control rats, the level of hydrogen peroxide radicals was elevated throughout the study.

DISCUSSION

Myocardial infarction and angina pectoris are serious diseases, which in some cases result in I/R injury, producing oxygen-derived free radicals that result in cardiac muscle cell death. Using H9c2 cells and rat hearts, we showed that pro-vitamin C protects against ROS induced injury in rats (Figs. 1 and 2). Interestingly, the combined pro-vitamin C and MEK inhibitor treatments did not reduce H_2O_2-induced injury more extensively than the pro-vitamin C treatment alone (Figs. 1 and 2). In addition, the H_2O_2-induced P-MAPK was moderately prevented by administration of Asc2P, and prevented to below detection level by the MEK inhibitor treatment (Fig. 1C).

Some ROS-associated biological phenomena have been shown to be effectively controlled by pro-vitamin C but not Asc, although the mechanism remains to be determined. In this study, pro-vitamin C protected against H_2O_2-induced injury of the H9c2 cells (Fig. 1A). Without pro-vitamin C, apoptotic cell death of the H9c2 cells resulted from H_2O_2-induced injury (Fig. 1D). Apoptotic cell death in the rat heart also resulted from I/R-induced injury (data not shown). In both cases, the apoptotic cell death was induced by ROS [Eguchi et al., 2003a]. Therefore, pro-vitamin C both protected against H_2O_2-induced cell injury and inhibited hydrogen peroxide radicals (Fig. 2). However, the combination of pro-vitamin C and MEK inhibitor also inhibited hydrogen peroxide radicals (Fig. 2C). In this case, why did the combination of pro-vitamin C and MEK inhibitor fail to protect against injury by I/R (Fig. 2)? It is suggested that prolonged ischemia causes necrosis, in which case, apoptosis was induced by myocardial infarction and angina pectoris. The P-MAPK is blocked by PKC inhibitors and phosphoinositide 3-kinase inhibitors (PI3K) [Mizukami et al., 2000].

Cell survival has been shown to be inhibited by inhibition of dependent on PI3K activity [Dudek et al., 1997]. Importantly, the MAPK pathway is activated by PI3K.

The P-MAPK was increased by H_2O_2 (200 μM) introduced in the H9C2 cells (Fig. 1C). This H_2O_2-induced activity of P-MAPK was prevented by administration of Asc2P, and the MEK inhibitor.

The activity of MAPK is known to take various pathways in live mammalian cells. Ischemia causes MAPK and JNK expression in the nucleus, and reperfusion causes activation [Mizukami et al., 1997]. We showed that MAPK was more activated by 100 μM H_2O_2 than by 500 μM H_2O_2 concentration, which injured the cell in the rat heart [Eguchi et al., 2003b]. Our findings also suggested that the Asc may be regulated by a pathway other than the MAPK pathway and the activity of the MAPK pathway may prevent H_2O_2 from producing ROS, when pro-vitamin C is administered.

In addition, it was suggested that theH9C2 cells require the activity of MAPK against H_2O_2-induced injury even when pro-vitamin C is administered, and that I/R injury in rat heart also requires MAPK activity (Fig. 2).

The MAP kinase family is regulated by effective cardiac muscle [Bogoyevitch et al., 1996]. Asc inhibits ROS, thereby protecting against ROS-induced injury; it was also suggested that the same pathway may increase cell survival.

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