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FAST TRACK

Cytoprotection by Pro-Vitamin C Against Ischemic Injuries in Perfused Rat Heart Together With Differential Activation of MAP Kinase Family

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Abstract The cardiac muscle cells are known to be killed by ischemia-reperfusion (I/R) treatment that produce reactive oxygen species (ROS). We analyzed the function of the autooxidation-resistant pro-vitamin C, 2-O-alpha-D-glucosylated derivative (Asc2G) of ascorbic acid (Asc), in protecting against I/R injury of the heart in rat. The serum release of the intracellular enzyme CPK due to I/R injury decreased upon injection with Asc2G. Out of the mitogen-activated protein (MAP) kinase family members, MAP kinase and JNK underwent the down-regulation in contrast to up-regulation of p38 compared with the I/R-treated control in the absence of Asc2G. These data suggest important roles for differential activation of the MAP kinase family in cytoprotection against I/R injury by Asc2G. J. Cell. Biochem. 89: 863–867, 2003.

Key words: pro-vitamin C; ischemia-reperfusion; MAP kinase family

Nutrition is supplied to the whole body through the organ of heart, but the components of cardiac muscle themselves need nutrition. This blood in the heart can get clogged and may produce myocardial infarction and angina pectoris. These are serious diseases, which in some cases result in I/R injury, producing oxygen-derived free radicals that result in cardiac muscle cell death. Prolonged ischemia causes necrosis and contractile dysfunction in the heart. However, the heart can recover from 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]. It has been indicated that upstream kinases for mitogen-activated protein kinase (MAPK) are important regulators of nuclear transcription activity. MAPK is a serin/threonine protein kinase whose activity is up-regulated by MAPK/extracellular signal-regulated kinases (MEK)-catalyzed phosphorylation of tyrosin and therionine residues [Sturgill et al., 1988; Crews et al., 1992]. I/R injuries have been shown to act as upstream kinases for mitogen-activated protein kinase (MAPK) activation. It has been shown that the MAPK activation pathway is present in nuclei of ischemic hearts [Mizukami and Yoshida, 1997; Onishi et al., 1997]. Free radicals are generated by I/R injury. The free radicals that form, H_2O_2 and O_2^-, have been shown to be resolved by antioxidants such as vitamin C (L-ascorbic acid; Asc), which is one of the water-soluble vitamins, and is suggested as one of the preventive or potentially cytocidal agents against cancer [Maramag et al., 1997; Nagao et al., 2000]. ROS generated upon I/R may be scavenged by Asc [Fujiwara et al., 1997; Kumano et al., 1998], because plasma lipoproteins exposed to aqueous peroxy radicals

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undergo no hydroperoxidation until depletion of endogenous Asc, which is depleted more promptly than other plasma ROS-scavengers such as SH groups, alpha-tocopherol, bilirubin, and urate. This suggests that Asc would be the most efficient in preventing oxidative damage [Frei et al., 1989; Kanatate et al., 1995]. However, Asc reacts with the oxidized vitamin C, dehydroascorbic acid, by ROS in vivo. Recently, the autooxidation-resistant pro-vitamin C, Asc2G, was shown to resist oxidized vitamin C. Intracellular Asc derived from Asc2G and Asc2P was also shown to be retained within the cell longer than that from administered Asc [Tsukaguchi et al., 1999]. Asc2G was scarcely converted to vitamin C by α-glucosidase, and Asc was transported by Asc transporters into cells [Saitoh et al., 1997; Kageyama et al., 1999].

In the present study, we show that Asc2G protected the rat heart against I/R injury. Using the Langendorff mode, activation of the MAP kinase family was examined in the rat heart, along with the function of Asc2G as a very promising antioxidant.

MATERIALS AND METHODS

Animals

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).

Perfusion Protocol

The hearts were prepared for Langendorff mode as described previously [Yoshida et al., 1993]. The heart was perfused with filtered Krebs–Henseleit buffer containing 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgCl_2$, 1.25 KH_2PO_4, 25 mM $NaHCO_3$, 118 mM NaCl, and 10 mM glucose. The protocol for the anti-oxidant study were performed; we added 50 mM Asc in the buffer while the controls had none. The buffer was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. The heart was perfused at a constant perfusion pressure of 50 mm/Hg.

I/R-Induced Injury in the Heart

The hearts were prepared for Langendorff mode. The buffer was maintained at 37°C and gassed with 95% N_2 and 5% CO_2. 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 CPK-HA test (Wako, Osaka) at indicated times after reperfusion. The activity of MAP kinase in the heart was measured on stock frozen soon 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 β-glycerophosphate, and 2.5 mM sodium pyrophosphate) homogenizer (Nichionrikaseisakusyo; 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 X100 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 Westen 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) in Tris-buffered saline containing 0.05% Tween-20 (Wako, Tokyo) and rat antibody. The following were added, and the mixture was kept at room temperature for 45 min: ERK 1 (K-23), JNK 1 (C-17), p-JNK (G-7) (Santa Cruz, California, USA), p-MAPK, p38, and P38 (New England Biolab, Massachusetts, USA). Horseradish peroxidase and anti-rabbit IgG (Amersham Pharmacia Biotech, New Jersey,

USA) were added to a secondary antibody at 4°C over night. The reactive bands were detected using enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

RESULTS

Effects of Asc in perfusates of the isolated rat heart on extracellular release of CPK in the Langendorff's perfusion apparatus.

After I/R injury in the rat heart, release of CPK into the buffer decreased with the use of Asc2G (Fig. 1). The release of CPK into the buffer prepared for Langendorff mode took place at 240 min after both 15 and 40 min ischemia (data not shown). 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. Even at 30 min of ischemia, Asc2G administered by i.v. to the rat prior to death decreased CPK release (data not shown). These data indicate that Asc2G protected against I/R injury in the rat heart.

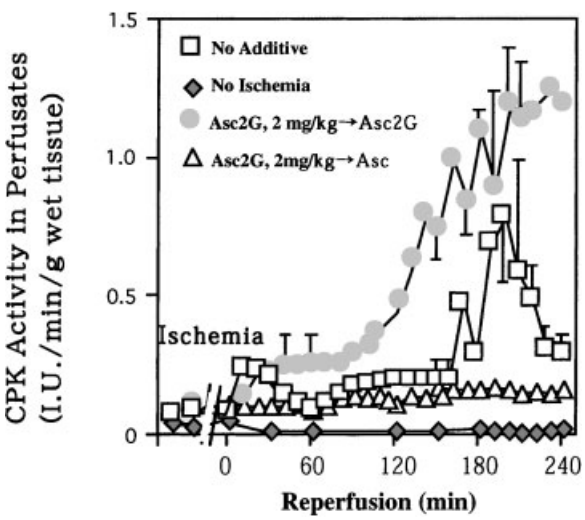

Fig. 1. Effects of ascorbic acid (Asc) in perfusates of the isolated rat heart on extracellular release of CPK in the Langendorff's perfusion apparatus. The rat underwent intravenous administration with or without Asc2G at a dose of equivalent to 2 mg Asc/kg. After 120 min, the heart was isolated and 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 with or without Asc or Asc2G at 50 μM in the perfusate. The release of CPK from the heart into the perfusate was quantified as an indicator for cytolysis of the heart at graded times of post-I/R. The data shown were typical of three to six independent experiments, respectively. The bar represents the SD of measurement points in triplicate in each experiment.

Activity of MAP Kinase Family

The phosphorylation of MAPK was increased by I/R injury in the nuclear fraction. The phosphorylation of JNK was activated by reperfusion at 15 min, while that of p-38 was activated by reperfusion at 0 min in the nuclear fraction. In contrast, the CPK release was decreased by Asc2G, and the phosphorylation of MAPK and JNK and p-38 in the nuclear fraction with Asc2G were more activated than control (Fig. 2). In the cytosol fraction of the heart, the phosphorylation of MAPK and JNK were increased by I/R injury in the rat heart. In the fraction containing Asc2G, CPK release was decreased, the phosphorylation of MAPK and p-38 was increased by I/R injury in the rat heart, and the phosphorylation of JNK was not increased by I/R injury in the rat heart (Fig. 2).

In this study, localization of Histon 1 in the nuclear fraction of the rat heart was increased by ischemia (data not shown). In contrast, Histon 1 in the nuclear fraction of Asc2G treated rat hearts was increased more after ischemia than did the control (data not shown). These data indicate that the Asc2G regulated the differential activation of MAP kinase family for protection against I/R injury.

DISCUSSION

Asc2G protected the heart from I/R injury, in which xanthine oxidase produces ROS; therefore, Asc2G inhibited ROS. In the present study, allopurinol as an inhibitor of xanthine oxidase was administered orally to rats whose hearts were protected from CPK release prepared by Langendorff mode perfusion I/R injury (data not shown). In addition, I/R injury is caused by calcium entry into the cell; we showed that allopurinol as a calcium channel blocker administered orally to rats protected against CPK release in the hearts prepared by Langendorff mode (data not shown). It was suggested that CPK release in rat hearts prepared for Langendorff mode caused xanthine oxidase, producing ROS [Hashimoto et al., 1994].

I/R injury was prevented when Asc was maintained at serum concentrations; Asc concentration has been shown to be decreased by I/R injury of the rat brain [Sato and Hall, 1992; Wilson and Jaworski, 1992]. The Asc2G was administered by i.v. before Langendorff mode; α-glucosidase almost converted all of it to vitamin C in rat serum about 120 min

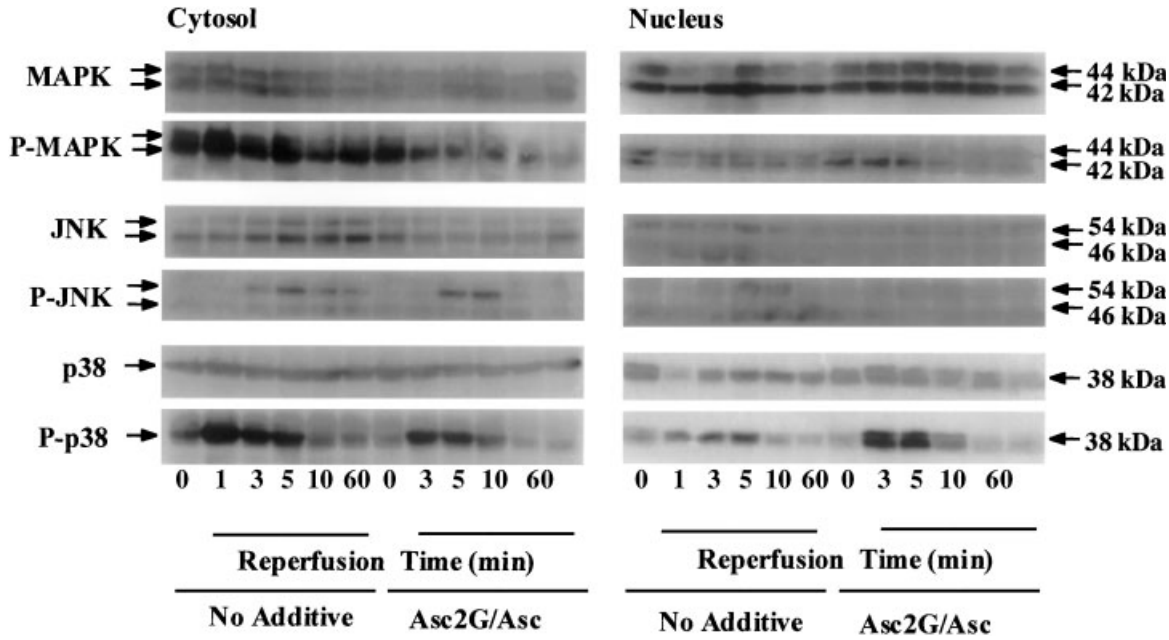

Fig. 2. Effects of Asc2G on alteration and phosphorylation of MAP kinase family members, MAPK, JNK, and p38, in the isolated rat heart subjected to I/R. The heart was subjected to I/R and administration with or without Asc2G in the same manner as in Figure 1. At graded times after outset of reperfusion, the nuclear and cytosol fractions of the perfused heart were separated and assessed by Western blots.

[Yamamoto et al., 1990]. It was suggested that Asc2G inhibited ROS from I/R injury, and Asc concentration and maintenance were necessary in serum [Eguchi et al., 2003].

MAPK and JNK were expressed in the nucleus by ischemia, and were activated by reperfusion [Mizukami et al., 1997]. In the present study, MAPK was increased in the nuclear fraction depending on the duration of the ischemia (data not shown). Although MAPK was activated in the control, the activity of MAPK is known to take various pathways in live mammalian cells. We showed that the MAP kinase family was activated by I/R injury in rat hearts through the Langendorff mode. In the ischemia at 40 min after reperfusion model, MAPK was less activated than in ischemia at 15 min. In addition, MAPK was more activated by the buffer prepared for the Langendorff mode containing 100 μM H_2O_2 than by that containing 500 μM H_2O_2 concentration, which injured the cell (data not shown). It was suggested that the activity of MAPK and ischemia injury follows a law of reciprocity [Sundaresan et al., 1995; Wei et al., 2001].

It was suggested that protection against I/R injury in the heart may need the activity of MAPK in the nucleus; with Asc2G administered, the activity of MAPK in the nucleus was maintained (Fig. 2).

JNK was expressed in the nuclear fraction by ischemic or stable conditions; therefore, it was more activated than MAPK upon I/R injury (Fig. 2). In addition, JNK was activated by the buffers prepared for the Langendorff mode containing either 100 or 500 μM H_2O_2 (data not shown).

However, the cell was injured by 500 μM H_2O_2 and was not injured by 100 μM H_2O_2. These data indicate that the p38 was expressed in the nuclear fraction by non-ischemic or stable conditions. However, the phosphorylation of p38 was expressed in the nuclear fraction by I/R injury (Fig. 2). In addition, the phosphorylation of p38 was activated by the buffer containing 100 μM H_2O_2 prepared for the Langendorff mode. However, the p38 in the nuclear fraction was not changed, and p38 was not activated by 500 μM H_2O_2 (data not shown). These data indicate that p38 was not effective against ROS produced by I/R injury in rat hearts. It was suggested that p38 was activated by a stimulation of osmotic pressure in the cell against ischemia [Bogoyevitch et al., 1996]. It was also suggested that the substrate of activity of p38 was the activity of MAPK–APK2

stimulated by the heat shock proteins [Stokoe et al., 1992].

In the present study, it was suggested that Asc protected against I/R-injury of the heart when the MAP kinase family was regulated by effective cardiac muscle [Bogoyevitch et al., 1996]. In sum, Asc2G is a substance that can be analyzed as having a new vitamin C function in the body and should excel as a new anti-oxidant drug.

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