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ORIGINAL ARTICLE

Geranylgeranylacetone and volatile anesthetic-induced cardiac protection synergism is dependent on caveolae and caveolin-3

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Abstract
Purpose Pharmacological preconditioning, including that with geranylgeranylacetone (GGA) and volatile anesthetics, has been shown to confer cardiac protection from ischemia/reperfusion injury although the mechanisms for this protection are poorly understood. Caveolins, integral membrane proteins that act as scaffolding proteins in caveolar membranes, localize molecules involved in cardiac protection. We have tested the hypothesis that caveolin-3 (Cav-3), the predominant isoform in cardiac myocytes, is essential for the synergistic effect observed between GGA and volatile anesthetics.
Methods Mice were randomly assigned to receive GGA, isoflurane [0.5 and 1.0 minimum alveolar concentration (MAC)], or GGA + isoflurane (0.5 MAC). An in vivo mouse model of ischemia/reperfusion injury was tested in wild-type and Cav-3 knockout mice, and the infarct size was determined. Biochemical assays were also performed in excised hearts.
Results Geranylgeranylacetone and therapeutic isoflurane (1.0 MAC) independently reduced infarct size (31.6 ± 6.1 and 28.0 ± 5.0 % of the area at risk, respectively; $n = 10$) as compared to the controls (45.8 ± 9.4 %; $n = 10$). The combination GGA + sub-therapeutic isoflurane (0.5 MAC) further decreased the infarct size to 19.3 ± 5.1 % ($n = 10$). Preconditioning [GGA, isoflurane (1.0 MAC), and GGA + isoflurane] increased the amount of Cav-3 protein in the discontinuous sucrose-gradient buoyant fractions. Additionally, cardiac protection was not observed in Cav-3 knockout mice following the administration of GGA, isoflurane, and GGA + isoflurane.
Conclusions Combined administration of GGA + isoflurane had a synergistic effect, enhancing the protection against myocardial infarction to a greater extent than either drug alone. This beneficial effect is mediated by Cav-3 expression.

Keywords Geranylgeranylacetone · Isoflurane · Caveolin-3 · Preconditioning · Cardiac protection

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Introduction

Myocardial ischemia/reperfusion injury is one of the leading causes of morbidity and mortality in the perioperative period. In both in vivo and in vitro settings, myocardial ischemia/reperfusion injury can be attenuated using a variety of interventions (termed "preconditioning"), such as brief episodes of ischemia [1], opioids [2, 3], and the noble gases xenon and helium [4, 5]. It has also been reported that the administration of volatile anesthetics, including isoflurane, can improve myocardial function and reduce myocardial infarct size following ischemia/reperfusion [6].

Caveolae are small flask-like invaginations of the sarcolemmal membrane and are enriched with lipids (e.g., cholesterol and glycosphingolipid) and signaling molecules. Caveolins are the structural proteins of caveolae and

are present in three isoforms, namely, caveolin-1, -2, and -3. Caveolin-3 (Cav-3) is specifically expressed in cardiac and striated muscles [7, 8]. We have recently shown that both caveolae and Cav-3 are key and essential components in ischemic [9, 10], anesthetic [11–13], and opioid [14] -induced myocardial preconditioning.

It has recently been reported that the oral administration of geranylgeranylacetone (GGA), a popular anti-ulcer drug in Asia, can protect the heart against ischemia/reperfusion injury by mediating the activation of protein kinase C and heat shock protein [15, 16]. Based on their experimental results, Kitahata et al. suggested that volatile anesthetics and GGA have an additive cardio-protective effect [17]. However, the exact mechanism of this synergism is still unclear. Thus, the aim of our study was to determine whether GGA- and isoflurane-induced cardiac protection is dependent on Cav-3 expression.

Materials and methods

Animals

All animals were treated in compliance with the Guidelines for Proper Conduct of Animal Experiment and Related Activities (Ministry of Education, Culture, Sports, Science and Technology of Japan) and the Guidelines for Care and Use of Lab Animals at the University of Tokushima. All animal use protocols were approved by the Animal Care and Use Committee at the University of Tokushima. Male C57BL/6 mice (8–10 weeks old, 21–26 g body weight) were purchased from Japan SLC, and Cav-3 knockout (Cav-3 KO) mice were bred as reported previously [18]. The animals were kept on a 12/12-h light–dark cycle in a temperature-controlled room.

Experimental preparation

Mice were randomly assigned to the experimental protocols. GGA was provided by Eisai Co. (Tokyo, Japan) and was administered to the mice by gavage at a dose of 200 mg/kg (dissolved with 0.4 % lecithin in deionized water). Mice in the control group were administered the same dose of vehicle. Twenty-four hours following treatment the mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) (Fig. 1). Surgical methods were similar to those previously described [19]. Briefly, a twenty-gauge catheter was inserted into the tracheae and mice were mechanically ventilated using a pressure-controlled ventilator (TOPO Ventilator; Kent Scientific Co., Torrington, CT). A thoracotomy was performed to expose the heart. Core temperature was maintained with a heating pad, and electrocardiogram leads were placed to record heart rate. The hemodynamic effects were measured through the right carotid artery cannulation with a 1.4 F Mikro-tip pressure transducer (model SPR-671; Millar Instruments, Houston, TX), which was connected to an amplifier (model TC-510; Millar Instruments) for the determination of heart rate, arterial blood pressure, and rate pressure product, as described previously [20].

Ischemia reperfusion protocol and experimental groups

A baseline was established after the thoracotomy, and mice were randomized to receive 100 % O_2 or 0.7 or 1.4 % isoflurane [1.4 % = 1.0 minimum alveolar concentration (MAC) for mice] [21]. Lethal ischemia was produced by occluding the left coronary artery with a 7-0 silk suture on a taper BV-1 needle (Ethicon Inc., Somerville, NJ) for 30 min. A small piece of polyethylene tubing was used to secure the silk ligature without damaging the artery. After 30 min of occlusion, the ligature was released and the heart reperfused for 2 h (Fig. 1).

Determination of infarct size

After 2 h of reperfusion, the coronary artery was once again occluded and the area at risk (AAR) determined by staining with 1 % Evans blue (1.0 mL; Sigma, St. Louis, MO). The heart was immediately excised and placed in 1 % agarose and allowed to harden. Once hardened, the heart was cut into 1-mm slices (McIlwain tissue chopper; Brinkmann Instruments, Inc., Westbury, NY). Each slice of left ventricle (LV) was counterstained with 3.0 mL of 1 % 2,3,5,-triphenyltetrazolium chloride (Sigma) for 5 min at 37 °C. After overnight storage in 10 % formaldehyde, the slices were weighed and visualized under a microscope (model SZ61-TR; Olympus, Tokyo, Japan) equipped with a charge coupled device camera (DXM 1200F; Nikon Corp.,

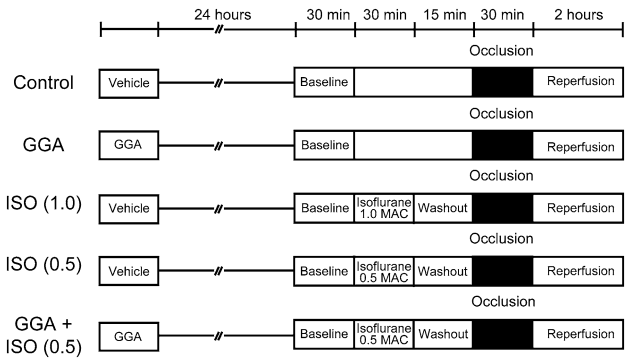

Fig. 1 Schematic illustration of the experimental protocol. *GGA* Geranylgeranylacetone, *MAC* minimum alveolar concentration, *ISO* (*1.0*) 1.0 MAC isoflurane, *ISO* (*0.5*) 0.5 MAC isoflurane, *GGA + ISO* (*0.5*) combination GGA + 0.5 MAC isoflurane

Tokyo, Japan). The images were then analyzed (Image-Pro Plus; Media Cybernetics, Rockville, MD), the AAR and infarct size (IS) were determined by planimetry as previously described [22]. The AAR was expressed as a percentage of the LV (AAR/LV), and IS was expressed as a percentage of the AAR (IS/AAR).

Sucrose density fractionation

Whole LVs were used for sucrose density membrane fractions, as reported previously [23]. After centrifugation, samples were removed in 1-mL aliquots to yield 12 fractions. We defined fractions 4–6 as buoyant membrane fractions enriched in caveolae and proteins associated with caveolae; fractions 9–12 were defined as nonbuoyant fractions.

Immunoblot analysis

Proteins in the membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10 % polyacrylamide precast gels (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane by electroelution. Membranes were blocked in phosphate buffered saline containing 2.0 % nonfat dry milk and incubated with primary antibody overnight at 4 °C (Cav-3; Santa Cruz Biotechnology, Santa Cruz, CA/Cav-3; BD Biosciences, San Jose, CA). Bound primary antibodies were visualized using secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology and ECL reagent from Amersham Pharmacia Biotech (Piscataway, NJ). All displayed bands migrated at the appropriate size, as determined by comparison to molecular weight standards (Santa Cruz Biotechnology).

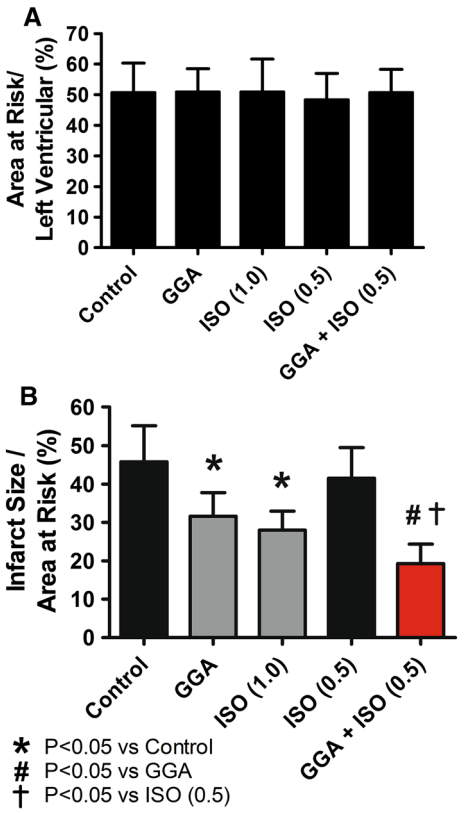

Fig. 2 **a** Area at risk (AAR) for myocardial ischemia as a percentage of the left ventricle did not differ among groups. **b** Myocardial infarct size expressed as a percentage of the AAR. $n = 10$ mice per group

Table 1 Hemodynamic parameters

Hemodynamic parameters	Pre-occlusion	Ischemia for 30 min	Reperfusion for 120 min
Heart rate (beats min^{-1})			
Control	436 ± 68	382 ± 38	370 ± 32
GGA	429 ± 56	411 ± 47	401 ± 40
ISO (1.0)	421 ± 40	408 ± 33	391 ± 25
ISO (0.5)	441 ± 63	394 ± 25	373 ± 36
GGA + ISO (0.5)	425 ± 51	415 ± 53	401 ± 28
Mean arterial pressure (mmHg)			
Control	71 ± 6	68 ± 5	63 ± 6†
GGA	71 ± 6	70 ± 5	68 ± 7§
ISO (1.0)	69 ± 7	69 ± 6	71 ± 7§
ISO (0.5)	68 ± 7	66 ± 4	60 ± 6
GGA + ISO (0.5)	72 ± 7	68 ± 4	70 ± 6§
Rate-pressure product (beats min^{-1} mmHg 10^3)			
Control	30.8 ± 4.9	25.9 ± 2.7	23.3 ± 3.1†
GGA	30.4 ± 3.4	28.5 ± 3.7	27.4 ± 4.2§
ISO (1.0)	29.0 ± 2.0	28.0 ± 3.4	28.0 ± 4.0*,§
ISO (0.5)	30.4 ± 6.2	26.0 ± 2.1	22.2 ± 2.7†
GGA + ISO (0.5)	31.1 ± 5.9	28.2 ± 4.2	28.0 ± 3.0*,§

Data are expressed as mean ± standard deviation (SD)

GGA geranylgeranylacetone, *ISO (1.0)* 1.0 MAC isoflurane, *GGA + ISO (0.5)* combination of GGA + 0.5 MAC isoflurane

* Significantly different from Control (intergroup comparison) at $P < 0.05$

§ Significantly different from 0.5 minimum alveolar concentration (MAC) isoflurane [ISO (0.5)] (intergroup comparison) at $P < 0.05$

† Significantly different from pre-occlusion (intragroup comparison) at $P < 0.05$

Statistical analysis

Statistical analyses were performed by one-way and two-way analysis of variance for repeated measures, followed by the Bonferroni post hoc test or unpaired Student's *t* test. All data are expressed as the mean ± standard deviation (SD). Statistical significance was defined as $P < 0.05$.

Results

For each group, the AAR compared with total LV weight was calculated, and no significant differences were observed between groups (Fig. 2a). There were also no significant differences between groups in terms of pre-occlusion heart rate, blood pressure, or rate pressure product (Table 1). Compared to the infarct size in the control mice at 2 h after infusion (45.8 ± 9.4 % of AAR; $n = 10$), that in mice treated with GGA or 1.0 MAC isoflurane was significantly reduced [31.6 ± 6.1 % ($n = 10$) or 28.0 ± 5.0 % ($n = 10$), respectively], while mice treated with 0.5 MAC isoflurane were not protected (41.6 ± 7.9 %; $n = 10$, Fig. 2b). However, the administration of GGA pretreatment in combination with 0.5 MAC isoflurane led to a yet further reduction in myocardial infarct size [19.3 ± 5.1 % ($n = 10$); $P < 0.05$ vs. GGA or vs. 0.5 MAC isoflurane].

We used biochemical techniques to fractionate the excised hearts on a discontinuous sucrose gradient and subsequently analyzed the fractions for distribution of Cav-3. Exposure of mice solely to sub-therapeutic 0.5 MAC isoflurane did not increase the amount of Cav-3 in the buoyant fractions, as assessed by sucrose density fractionation. In contrast, adminstration of GGA, 1.0 MAC isoflurane, and GGA + low-dose isoflurane (0.5 MAC) did increase the amount of Cav-3 protein in the buoyant fractions (Fig. 3a, b; $n = 5$). Additionally, the expression of Cav-3 was higher in the buoyant fractions receiving the combined GGA pretreatment + 0.5 MAC isoflurane than in those treated with either GGA or isoflurane alone (Fig. 3a, b).

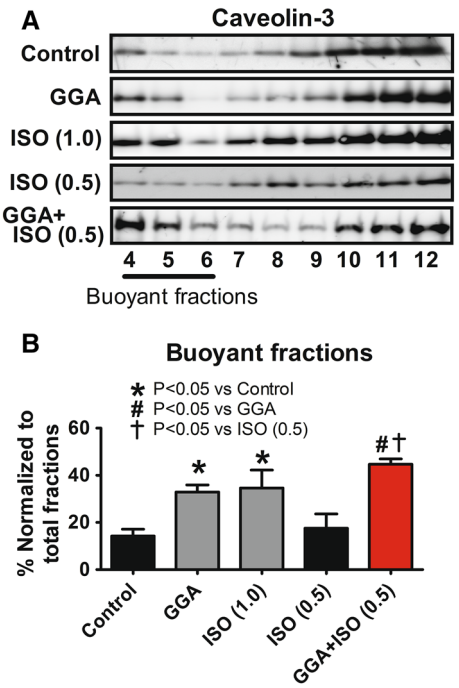

Fig. 3 Lysed and fractionated hearts on a sucrose density gradient. Fractions were collected and probed for caveolin-3. Significant localization of caveolin-3 in the buoyant fractions was observed in the groups treated with GGA, ISO (1.0), and GGA + ISO (0.5), whereas Control mice and mice treated with ISO (0.5) mice showed no effects on caveolin-3 localization

We found no significant differences between wild-type (WT) and Cav-3 KO mice in pre-occlusion heart rate, blood pressure, or rate pressure product, both with and without GGA/isoflurane administration, throughout the procedure (Table 2). Occlusion and post-occlusion hemodynamics were also not significantly different among the groups (data not shown). The AAR was calculated as a percentage of the LV mass and was similar between groups (data not shown). The administration of GGA, therapeutic isoflurane (1.0 MAC), or GGA + low dose isoflurane (0.5 MAC) did not have a protective effect in Cav-3 KO mice (42.1 ± 5.1, 41.2 ± 8.5, or 40.2 ± 10.7 %, respectively; $n = 8$ per group; Fig. 4).

Discussion

Our data suggest that isoflurane-induced cardiac protection is dose dependent and that the expression of Cav-3 is essential in GGA- and isoflurane-induced cardiac protection. We also show that this protection can be enhanced when GGA is paired with sub-therapeutic isoflurane treatments. Consistent with these findings, the protective effects of these pharmacological agents were abolished in Cav-3 KO mice, indicating that the observed protection is mediated by Cav-3 expression.

Volatile anesthetics, including halothane, isoflurane, and sevoflurane, have been shown to exert cardiac protective effects when administered prior to coronary artery occlusion, a phenomenon termed anesthetic-induced preconditioning [6, 24, 25]. Previous studies have suggested that anesthetic-induced preconditioning involves the activation of similar pathways involved in ischemic preconditioning [26, 27], with the activation of Src tyrosine kinase, the phosphatidylinositol-3-kinase/protein kinase B/glycogen synthase kinase 3 beta pathway, protein kinase C (PKC), mitogen-activated protein kinases, and mitochondrial and sarcolemmal adenosine triphosphate-regulated potassium channels [28], all of which are associated with caveolins [29]. Interestingly, these protective effects are completely absent in Cav-3 KO mice, suggesting that Cav-3 is essential to the regulation of these mechanisms [9, 13, 14]. In a previous study we showed that Cav-3 has a temporal and spatial importance and that it is perhaps one of the initial and vital components necessary for cell membrane to organelle communication [30].

In this study with our mouse model, we observed that exposure to 1.0 but not 0.5 MAC isoflurane reduced infarct

Table 2 Hemodynamic parameters at pre-occlusion period

Hemodynamic parameters	Caveolin-3 knockout mice				
	Control	GGA	ISO (1.0)	ISO (0.5)	GGA + ISO (0.5)
Heart rate (/min)	447 ± 37	426 ± 40	445 ± 59	427 ± 50	438 ± 28
Mean arterial pressure (mmHg)	71 ± 7	74 ± 3	71 ± 5	69 ± 8	73 ± 4
Rate–pressure product (beats min^{-1} mmHg 10^3)	31.7 ± 4.6	31.7 ± 3.5	31.6 ± 4.6	29.5 ± 4.4	32.0 ± 3.3

Data are expressed as the mean ± SD

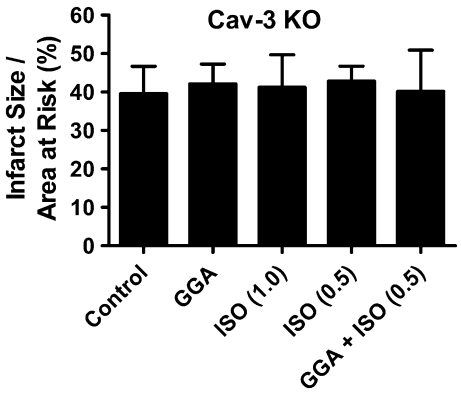

Fig. 4 Myocardial infarct size expressed as a percentage of the left ventricular AAR. Caveolin-3 knockout (Cav-3 KO) mice were not protected with GGA- and/or ISO-preconditioned treatments. $n = 8$ per group

size as compared with the control. We and other investigators have previously shown that volatile anesthetics have dose-dependent protective effects [17, 31–34]. Using a dog model, Kehl et al. [35] reported that 0.25 MAC isoflurane was sufficient for preconditioning the myocardium against infarction, but they also noted a dose-dependent cardioprotective effect. In contrast, Piriou et al. reported that in their rabbit model 1.0 MAC sevoflurane was insufficient to protect against myocardial infarction [36]. These findings suggest that the effects of anesthetic preconditioning may be dependent on the individual properties of volatile anesthetics, as well as the experimental animal model.

GGA has been shown to have a protective effect in multiple organs. In rat liver, the administration of GGA suppressed ischemia/reperfusion injury, inhibiting hepatocyte apoptosis caused by an enhanced heat shock response through the activation of heat shock factor 1. GGA has also been reported to have protective effects against delayed cerebral vasospasm after rat subarachnoid hemorrhage [37]. In isolated perfused rat hearts, Ooie et al. [15, 16] observed that the oral administration of GGA enhanced the expression of heat shock protein through the activation of PKC, resulting in improved myocardial function after ischemia. These authors also demonstrated preserved mitochondrial respiratory function, suggesting that GGA has a cardio-protective effect against ischemia/reperfusion [38]. More recently, we found that low dose volatile anesthetics (0.5 MAC) did not reduce myocardial infarct size compared with that in control animals; however, the cardioprotective effect of GGA was enhanced when those animals pretreated with GGA were exposed to 0.5 MAC of volatile anesthetics for 30 min before ischemia [17]. In the present study, we show that Cav-3 is essential for this synergistic effect between GGA and isoflurane.

Caveolae, cholesterol, and sphingolipid-enriched invaginations of the plasma membrane all play important roles in physiological function and are vital to cardiac protective mechanisms [29, 30]. Caveolae and caveolins regulate receptor stability, myocardial hypertrophy, signaling, calcium homeostasis, and endocytosis. Cav-3, the predominant isoform in cardiac myocytes, mediates interactions with cytoskeletal elements (including α-tubulin and filamin) and is responsible for caveolae formation in these cells [7, 8]. Caveolins have been shown to play a fundamental role in the phenomenon of myocardial preconditioning. Recent studies have shown that overexpression of cardiac Cav-3 can produce innate cardiac protection and enhance caveolar formation, whereas a decrease in the number of myocardial caveolae has been described in Cav-3 KO mice although Cav-1 levels did remain stable [14]. Additionally, these mice lose the ability to elicit preconditioning-like cardiac protection from ischemia/reperfusion injury in both in vitro and in vivo models [9, 10, 12–14]. These results imply that Cav-3 and the presence of caveolae are required for eliciting volatile anesthetic-induced cardiac protection from ischemia–reperfusion injury. Also, preconditioning may modulate the microenvironment of caveolae and caveolin-associated protein interactions to enable the enrichment of proteins that promote cardiac protection. This notion is consistent with findings indicating that endothelial nitric oxide synthase and the glucose transporter GLUT-4 translocate to caveolae after preconditioning [39]. Taken together, these data suggest that Cav-3 may have a significant clinical role in mediating multiple signaling mechanisms, as well as regulating adaptive protective processes, and that perhaps overexpression of Cav-3 may enhance these properties. In the future, targeting Cav-3 expression or caveolae formation may be a potential therapeutic strategy for providing the heart with protection from significant ischemic damage.

In summary, our findings suggest that GGA preconditioning increases Cav-3 expression and that this expression helps facilitate the temporal and spatial organization of protective mechanisms. Our results also support the hypothesis that Cav-3 activation is an essential signaling component in the synergism observed between GGA and isoflurane.

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