Role of the O-linked β-*N*-acetylglucosamine in the Cardioprotection Induced by Isoflurane

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ABSTRACT

Background: Cardiac protection by volatile anesthetic-induced preconditioning and ischemic preconditioning have similar signaling pathways. Recently, it was reported that augmentation of protein modified with O-linked β-*N*-acetylglucosamine (O-GlcNAc) contributes to cardiac protection. This study investigated the role of O-GlcNAc in cardiac protection induced by anesthetic-induced preconditioning.

Methods: O-GlcNAc-modified proteins were visualized by immunoblotting. Tolerance against ischemia or reperfusion was tested *in vivo* (n = 8) and *in vitro* (n = 6). The opening of the mitochondrial permeability transition pore (mPTP) upon oxidative stress was examined in myocytes treated with calcein AM (n = 5). Coimmunoprecipitation and enzymatic labeling were performed to detect the mitochondrial protein responsible for the mPTP opening.

Results: Isoflurane treatment and the consequent augmentation of O-GlcNAc concentrations reduced the infarct size (26 ± 5% [mean ± SD], $P < 0.001$) compared with the control. The protective effect of O-GlcNAc was eliminated in the group pretreated with the O-GlcNAc transferase inhibitor alloxan (39 ± 5%, $P < 0.001$). Myocyte survival also showed the same result *in vitro*. Formation of the mPTP was abrogated in the isoflurane-treated cells (86 ± 4%, $P < 0.001$) compared with the control and alloxan-plus-isoflurane–treated cells (57 ± 7%, $P < 0.001$). Coimmunoprecipitation and enzymatic labeling studies revealed that the O-GlcNAc–modified, voltage-dependent anion channel restained the mPTP opening.

Conclusions: Isoflurane induced O-GlcNAc modification of mitochondrial voltage-dependent anion channel. This modification inhibited the opening of the mPTP and conferred resistance to ischemia-reperfusion stress.

What We Already Know about This Topic

- Studies indicate that O-linked β-*N*-acetylglucosamine (O-GlcNAc) may be associated with ischemic preconditioning of cardiac myocytes
- The current study investigated whether anesthetic preconditioning induces an increase in myocardial protein modified with O-GlcNAc, resulting in cardioprotective effects against ischemia or reperfusion injury

What This Article Tells Us That Is New

- Isoflurane induced O-GlcNAc modification of mitochondrial voltage-dependent anion channel. This modification inhibited the opening of the mitochondrial permeability transition pore and conferred resistance to ischemia or reperfusion stress

O-LINKED β-*N*-acetylglucosamine (O-GlcNAc) is a posttranslational glycosylation modification of nucleocytoplasmic and mitochondrial proteins and is analogous to phosphorylation or acetylation of proteins.[1] This glycosylation of serine or threonine residues is catalyzed by O-linked β-N acetylglucosamine transferase (OGT), whereas β-D-*N*-acetylglucosaminidase catalyzes deglycosylation.[2] More than 80 different proteins, including transcription factors, kinases, and phosphatases, are reversibly modified by O-GlcNAc, which is necessary for cell survival,[3] and Zachara *et al.*[4] reported that multiple stress stimuli increased cellular concentrations of O-GlcNAc in mammalian cells. When this response was blocked, cell survival decreased, but when the concentrations of O-GlcNAc were increased, cell viability was maintained.[4] Studies indicate that O-GlcNAc also may be associated with ischemic preconditioning (IPC) of cardiac myocytes.[1,5]

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Received from The University of Tokushima Graduate School, Tokushima, Japan. Submitted for publication December 21, 2010. Accepted for publication July 5, 2011. Supported by a Grant-in-Aid for Young Scientists (B) 22791437 from the Japan Society for the Promotion of Science, Tokyo, Japan (to Dr. Hirose); a Grant-in-Aid for Young Scientists (A) 20591833 from the Japan Society for the Promotion of Science (to Dr. Tsutsumi); and the Takeda Science Foundation, Tokyo, Japan. Presented in part at the American Society of Anesthesiologists Annual Meeting, San Diego, California, October 16–20, 2010.

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 Anesthesiology 2011; 115:955–62

Several exogenous substances, including adenosine, bradykinin, noradrenalin, δ-opioid agonists, and inhalational volatile anesthetics, trigger preconditioning.[6–7] Cardiac protection induced by volatile anesthetics is known as anesthetic preconditioning (APC). APC signaling is complicated but is thought to correspond with IPC signaling. Both APC[8] and IPC[9] are reported to prevent opening of mPTP. Recently, O-GlcNAc modification of the mitochondrial voltage-dependent anion channel (VDAC) was reported to be critical for myocardial survival.[1] However, it is not known whether APC induces an increase in the abundance of O-GlcNAc modified proteins, especially VDAC, or whether such an increase would lead to protection against ischemic injury.

Thus, the hypothesis is that APC induces an increase in protein modified with O-GlcNAc in myocardia, resulting in cardioprotective effects against myocardial ischemia or reperfusion injury. In addition, isoflurane inhibits opening of mPTP during oxidative stress by way of increased O-GlcNAc modification of VDAC. To investigate these hypotheses, immunoblotting, *in vivo* and *in vitro* cell survival studies, fluorescence imaging with calcein AM, coimmunoprecipitation, and enzymatic labeling were used to examine O-GlcNAc–modified VDAC. The results show that the intramitochondrial signaling is necessary for isoflurane-induced cardiac protection against ischemia-reperfusion injury.

Materials and Methods

Animals

All animals were treated in compliance with the "Guide for the Care and Use of Laboratory Animals"[10] and animal use protocols approved by the University of Tokushima Graduate School, Institutional Animal Care and Use Committee (Tokushima, Japan). Male C57BL/6 mice (8–10 weeks old, 24–26 g body weight) were kept on a light–dark cycle (12:12 h) in a temperature-controlled room.

Immunoblot

To detect whether O-GlcNAc concentrations increased after APC induced by isoflurane or decreased after pretreatment with alloxan (σ, Sigma-Aldrich, St. Louis, MO), an OGT inhibitor immunoblotting study was performed (n = 3). Mice were subjected to APC with isoflurane for 30 min; some mice were pretreated with alloxan (50 mg/kg, IV) 20 min before exposure to isoflurane. Fifteen minutes after the isoflurane exposure, the hearts were removed. Proteins prepared from whole-tissue lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a membrane by electroelution. O-GlcNAc was detected using the O-GlcNAc Western Blot Detection Kit (Pierce/Thermo Scientific, Rockford, IL). Briefly, membranes were blocked in blocking buffer and incubated with primary antibody C-terminal domain of pol II (CTD) 110.6, which is a specific monoclonal antibody for the detection of O-GlcNAc. Bound primary antibodies were visualized using secondary antibodies conjugated with horseradish peroxidase for immunoglobulin M and enhanced SuperSignal West Dura Chemiluminescent Substrate (Wako Pure Chemical Industries, Kanagawa, Japan). Concentrations of O-GlcNAc in the entire lane were normalized to β-tubulin and expressed as a percentage of control (set at 100%).

In Vivo Myocardial Ischemia and Reperfusion Experiment

Surgical methods were similar to those described previously.[11–13] Mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) and mechanically ventilated with 100% oxygen using a pressure-controlled ventilator (TOPO Ventilator, Kent Scientific Co., Torrington, CT). Core body temperature was maintained with a heating pad, and electrocardiogram leads were placed to record the heart rate. Cardiac catheterization was performed with a high-fidelity 1.4-French microtip pressure transducer (SPR-671, Millar Instruments Inc., Houston, TX) for the determination of hemodynamics. Ischemia was produced by occluding the left coronary artery with a 7-0 silk suture for 30 min, after which the ligature was released and the heart reperfused for 2 h.

Mice were randomly assigned to one of four experimental groups (n = 8). All mice underwent a 30-min left coronary artery occlusion followed by 2 h of reperfusion. Mice received vehicle (0.9% saline, IV) or an inhibitor of O-GlcNAc transferase (alloxan 50 mg/kg, IV) 20 min before isoflurane exposure in the presence or absence of 1.0 minimum alveolar concentration isoflurane (1.4% for mice[14]) (fig. 1A).

After 2 h of reperfusion, the coronary artery was again occluded and the area at risk (AAR) was determined by staining with 1% Evans blue. The heart was immediately excised and cut into 1.0-mm slices (McIlwain tissue chopper; Brinkmann Instruments, Inc., Westbury, NY). Each slice of left ventricle was counterstained with 2,3,5,-triphenyltetrazolium chloride (σ, 1%), stored overnight in formaldehyde (10%), weighed, and visualized using a microscope equipped with a digital camera (DXM 1200, Nikon, Tokyo, Japan). Images were analyzed by Image-Pro Plus software (Media Cybernetics, Bethesda, MD), and infarct sizes were determined by planimetry. Cardiac troponin-I concentrations in serum were measured using a high-sensitivity mouse cardiac troponin-I enzyme-linked immunosorbent assay kit (Life Diagnostics, West Chester, PA).

Simulated Ischemia or Reperfusion in Isolated Cardiac Myocytes

Adult C57BL/6J mice were anesthetized with pentobarbital (80 mg/kg, intraperitoneal) and the hearts were perfused using a Langendorff apparatus with Ca^{2+}-free heart media (in mM: 112 NaCl, 5.4 KCl, 1 $MgCl_2$, 9 NaH_2PO_4, and 11.1 D-glucose; supplemented with 10 HEPES, 30 taurine, 2 DL-carnitine, and 2 creatine, pH 7.4) for 5 min at 3 ml/min at 37°C, followed by perfusion with Ca^{2+}-free heart media

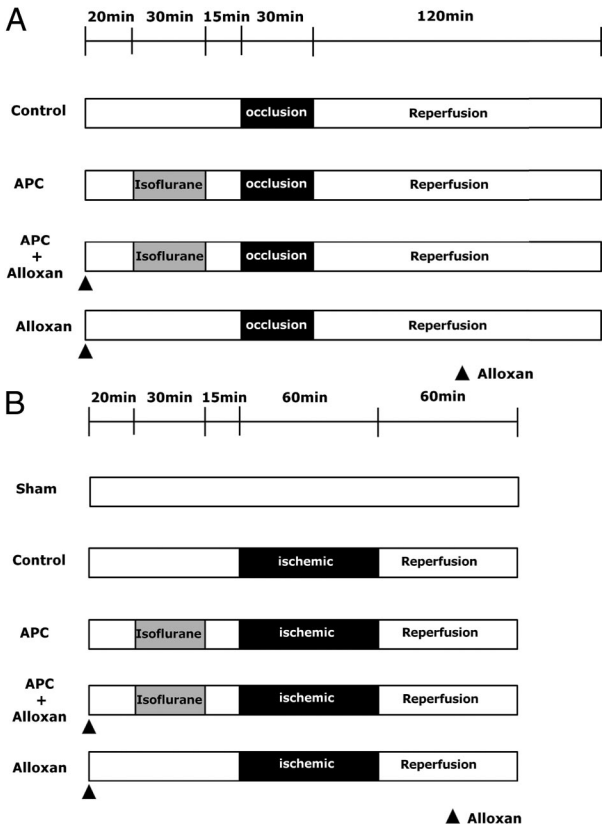

Fig. 1. Experimental protocols for *in vivo* studies. Mice were subjected to pretreatment with alloxan and/or the anesthetic preconditioning (APC) protocol (30 min isoflurane) followed by left anterior descending coronary artery occlusion for 30 min and 2 h reperfusion (n = 8 per group) *(A)*. Experimental protocols for *in vitro* studies. Cardiac myocytes were isolated and pretreated according to the protocol before exposure to simulated ischemia/reperfusion (n = 6 per group) *(B)*.

containing collagenase II (240 U/mg; Worthington Biochemical Corporation, Lakewood, NJ) for 20 min. After perfusion, both ventricles of the heart were removed and minced in heart media; the cell solution was washed to remove collagenase II and reexposed to 1.2 mM Ca^{2+} for 25 min to yield Ca^{2+}-tolerant cardiac myocytes, which were then incubated at 37°C in CO_2 (5%).

Cardiac myocytes were randomly distributed into five experimental groups (n = 6): sham; control; APC, in which cardiac myocytes were exposed to isoflurane (1.4%) for 30 min before the start of simulated ischemia and reperfusion; alloxan, an inhibitor of OGT; and APC plus alloxan. Twenty minutes before isoflurane exposure, 2.5 mM alloxan was added, as indicated (fig. 1B). Simulated ischemia was induced by a 95% N_2 and 5% CO_2 gas mixture and glucose-free media (glucose-free Dulbecco modified Eagle medium; Invitrogen, Carlsbad, CA) for 60 min. This was followed by 60 min of reperfusion by incubating the cells in 95% air and 5% CO_2. Cell viability was determined using trypan blue. Investigators were blinded to the treatment groups.

Opening of mPTP in Cardiac Myocytes

Inner mitochondrial membrane permeability to the fluorescent dye calcein AM indicates the opening of mPTP in intact cells.[15–16] Cardiac myocytes were loaded with the fluorescent probe calcein AM (1.0 μM; Invitrogen) and cobalt chloride (1.0 M; St. Louis, MO) for 30 min at 37°C. To induce oxidative mitochondrial damage, the fluorescent cardiac myocytes with internalized calcein AM were exposed to 100 μM H_2O_2 for 10 min. Calcein fluorescence in the cytosol is quenched by cobalt chloride, and staining is more diffuse when mPTP are closed than when mPTP are open. Myocytes were subjected to oxidative stress until mPTP opened, and the release of calcein from mitochondria was visualized using a microscope (Eclipse TE 2000-U; Nikon). Cells were illuminated with an argon-krypton laser (480 nm) and visualized using a 520-nm filter. The fluorescence intensity was scanned at 10-s intervals with Aquacosmos (Hamamatsu Photonics, Hamamatsu, Japan). The same experiment was performed separately to obtain the images of myocytes shown in the figure. Five samples from each group were examined.

Coimmunoprecipitation

Adult mouse cardiac mitochondrial proteins were examined to reveal O-GlcNAc associated with VDAC-1. Anti-O-GlcNAc antibody (RL2) or anti-VDAC antibody and 1ysis buffer were added to 50 μl of the sample and mixed. The samples were immunoblotted for VDAC using anti-VDAC (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA) or O-GlcNAc using RL2 (1:1,000, Affinity BioReagents, Golden, CO).

Enzymatic Labeling of O-GlcNAc–Modified Proteins

Glycosylation of VDAC was also assessed by immunoprecipitation and subsequent click labeling where O-GlcNAc–modified proteins were labeled using the Invitrogen Click-iT® O-GlcNAc Enzymatic Labeling System. The N-Azidoacetylgalactosamine, Acetylated–labeled O-GlcNAc–modified protein mixture was precipitated using chloroform-methanol, and the azide-labeled proteins were tagged with a fluorescent dye, tetramethylrhodamine-alkyne (Invitrogen). The dried-labeled protein sample was resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis buffer for electrophoresis, and the gel was visualized using a 532-nm laser equipped with a 580 blood pressure emission filter and a Typhoon 9400 imager (GE Healthcare Japan, Tokyo, Japan).

Staining for the Detection of Proteins

SYPRO® Ruby gel staining (Invitrogen) was used to show that equivalent amounts of total VDAC proteins were used for all samples from the experimental groups. The same gel used in Click iT labeling was stained for total protein using SYPRO® Ruby gel stain, destained, and visualized using a

488 laser, a 610 blood pressure 30-emission filter, and a Typhoon 9400 imager.

Statistical Analysis

Statistical power analysis revealed that a group size of n = 8 would provide sufficient power (0.8) to detect differences between means infarct size indices 15 (SD = 10; $\alpha = 0.05$).

A group size of n = 4 was used for *in vitro* and fluorescence experiments to provide a power of 0.8 to detect a difference between mean indices of 20 (SD = 10; $\alpha = 0.05$).

Data were analyzed using SPSS version 19 software (SPSS Inc., Chicago, IL). All data are expressed as mean ± SD, and statistical analyses of hemodynamic data were performed using two-factor, repeated-measures analysis of variance for time and treatment. If an overall difference between the variables was observed, comparisons were performed as a one-way analysis of variance followed by Newman-Keuls *post hoc* test for each group and for each time. Statistical analyses for Western blotting, *in vivo*, *in vitro*, and fluorescence studies were performed using one-way analysis of variance followed by Tukey *post hoc* test. The nature of the hypothesis testing is two-tailed testing. Statistical significance was defined as $P < 0.05$.

Results

O-GlcNAc Concentrations after APC

As shown in figure 2, cardiac O-GlcNAc concentrations were increased relative to those of control groups after APC. Densitometric analysis of each lane in the CTD110.6 blot shows 1.7-fold increase in the intensity of CTD110.6 immunostaining in isoflurane-treated heart relative to control heart (n = 3). However, pretreatment with alloxan reversed the effect of APC. Figure 2A shows multiple positive staining protein bands because many proteins are glycosylated.

Myocardial AAR and Infarct Size

Mice were assigned to one of four experimental protocols as described in figure 1A. No differences in baseline hemodynamics were observed among the experimental groups (table 1). Compared with the left ventricular weight, the AAR was similar among all groups, as shown in figure 3A. Two-hour reperfusion in control mice resulted in myocardial infarct size of 43 ± 3% (n = 8) of the AAR, and animals treated with isoflurane had myocardial infarct sizes that were 26 ± 5% (n = 8, F[3, 28] = 14.966, $P < 0.001$) of the AAR compared with those of controls (fig. 3B). Alloxan alone did not affect the size of the myocardial infarction; however, alloxan attenuated the protective effects of isoflurane treatment with respect to infarct size (39 ± 5% [n = 8, $P < 0.001$]; fig. 3B). Serum cardiac troponin-I, a marker of cardiac myocyte damage, was measured after 2 h of reperfusion, and the results confirmed the infarct size measurements (fig. 3C).

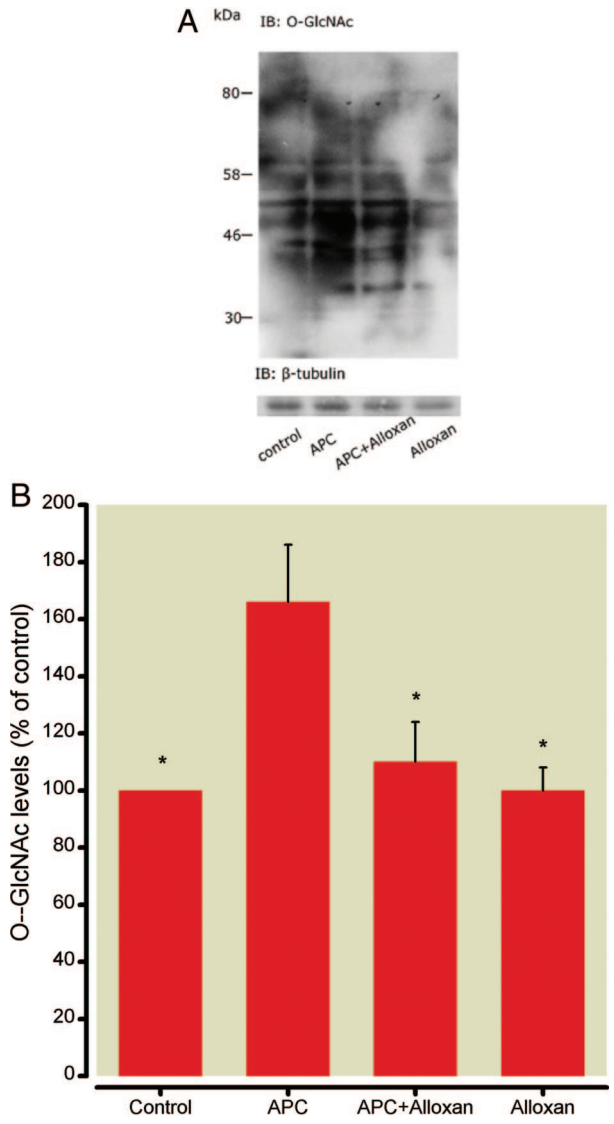

Fig. 2. Representative immunoblot (IB) of proteins from hearts subjected to anesthetic preconditioning (APC), pretreatment with alloxan alone, pretreatment with alloxan before APC, or sham. Multiple protein bands were detected, and each band represents a protein modified with O-linked β-*N*-acetylglucosamine (O-GlcNAc). APC increased the intensity of these bands compared with sham treatment and alloxan treatment alone, and the bands were eliminated with alloxan treatment *(A)*. Mean intensity of all O-GlcNAc proteins determined by densitometric analysis. Concentrations of O-GlcNAc were normalized to β-tubulin and expressed as a percentage of control samples *(B)*. * $P < 0.05$ *versus* APC. Mean ± SD, n = 3 per group.

Cell Survival after Simulated Ischemia-reperfusion

Simulated ischemia-reperfusion decreased cell viability to 53 ± 7% (n = 6, F[4, 25] = 10.858, $P = 0.004$) compared with the sham group (73 ± 7% [n = 6]; fig. 4), whereas pretreatment with isoflurane prevented cell death after simulated ischemia and reperfusion (71 ± 10% [n = 6, $P = 0.012$]; fig. 4). The viability of cells pretreated with alloxan alone was not different from the control ($P = 1.000$). How-

Table 1. Hemodynamic Measurements in Different Experimental Groups

	Tb	T0	T30	T120
Systemic BP (mmHg)	—	—	—	—
Control	69 ± 4	67 ± 3†	63 ± 3†	59 ± 4†
APC	71 ± 4	70 ± 3	68 ± 4†	65 ± 2*†
Alloxan	73 ± 7	71 ± 6	63 ± 4†	56 ± 5†
APC + Alloxan	75 ± 6	72 ± 10	59 ± 6†	55 ± 5†
Heart rate (beats/min)	—	—	—	—
Control	461 ± 24	443 ± 23	435 ± 22	426 ± 27
APC	444 ± 17	425 ± 12	421 ± 25	445 ± 26
Alloxan	448 ± 37	431 ± 47	434 ± 51	433 ± 47
APC + Alloxan	428 ± 18	432 ± 41	423 ± 48	415 ± 31

Values are expressed as mean ± SD.

* $P < 0.05$ compared with corresponding control. † $P < 0.05$ compared with baseline in the same group.

APC = anesthetic preconditioning; BP = blood pressure; Tb = baseline; T0 = before occlusion; T30 = 30 min from the start of occlusion; T120 = at the end of reperfusion.

ever, treatment of cells with alloxan before isoflurane reduced cell survival to 47 ± 12% (n = 6, $P = 0.001$). Treatment with alloxan abolished the protective effects of isoflurane on simulated ischemia-reperfusion.

Opening of mPTP in Cardiac Myocytes

Figure 5A shows a representative fluorescence image of calcein AM in cardiac myocytes at baseline and 10 min after exposure to 100 μM H_2O_2. The fluorescence intensity of cells from the isoflurane-treated group was greater (86 ± 4% of baseline [n = 5]) than that of both the control group (61 ± 9% of baseline [n = 5, F[3, 16] = 17.491, $P < 0.001$]) and the alloxan-plus-isoflurane group (57 ± 7% of baseline [n = 5, $P < 0.001$]). Pretreatment of cells with alloxan alone did not have protective or harmful effects. A time course of calcein intensity revealed delayed opening of mPTP in the isoflurane-treated group (fig. 5B). A summary of the data for calcein AM fluorescence of cardiac myocytes 10 min after exposure to 100 μM H_2O_2 is shown in figure 5C. The isoflurane-treated group reduced H_2O_2-induced mPTP opening in adult cardiac myocytes but not in cells pretreated with alloxan before exposure to isoflurane.

VDAC Is an O-GlcNAc Glycosylated Mitochondrial Protein

Because VDAC is a target for modification by O-GlcNAc, VDAC concentrations of cardiac myocytes were monitored after OGT inhibition to identity a link between O-GlcNAc signaling and mitochondria. Coimmunoprecipitation experiments showed that adult cardiac mitochondria from alloxan-plus-isoflurane–treated mice contained less O-GlcNAc–modified VDAC than did mitochondria obtained from

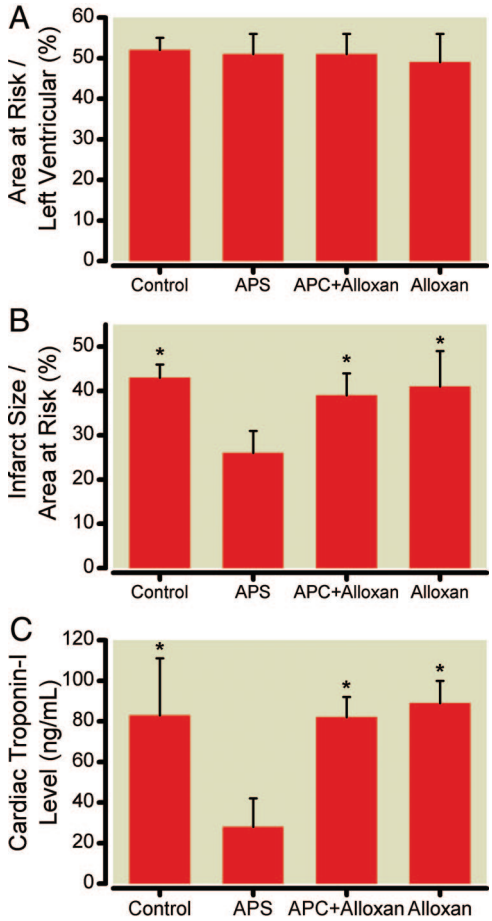

Fig. 3. The area at risk (AAR) compared with left ventricular weight was similar among all groups *(A)*. Infarct size expressed as a percentage of the AAR was reduced by anesthetic preconditioning (APC), but the effect was eliminated after pretreatment with alloxan. * $P < 0.05$ *versus* APC *(B)*. The release of troponin-I after 2 h of reperfusion indicates the magnitude of damage caused by ischemia-reperfusion. The magnitude of damage in the heart was larger in control, alloxan + APC, and alloxan groups than in the APC group. * $P < 0.05$ *versus* APC. Mean ± SD, n = 8 per group *(C)*.

isoflurane-treated mice and control mice (fig. 6A). Moreover, an alternative technique confirmed the coimmunoprecipitation findings (fig. 6B). SYPRO® ruby staining showed there was no detectable difference in total VDAC among the groups (fig. 6B). Together, the results shown in figure 6, A and B, revealed a greater degree of O-GlcNAc modification of VDAC in mice that had been treated with isoflurane than in the untreated control mice; however, this modification was precluded by alloxan pretreatment.

Discussion

Several conclusions can be drawn from this study: (1) APC in mice increases O-GlcNAc concentrations *in vivo*; (2) the elevation of O-GlcNAc concentrations induced by isoflurane is sufficient to reduce infarct size after *in vivo* myocardial ischemia-reperfusion injury; (3) O-GlcNAc modification enhances viability of cardiac myocytes after APC *in vitro*; (4)

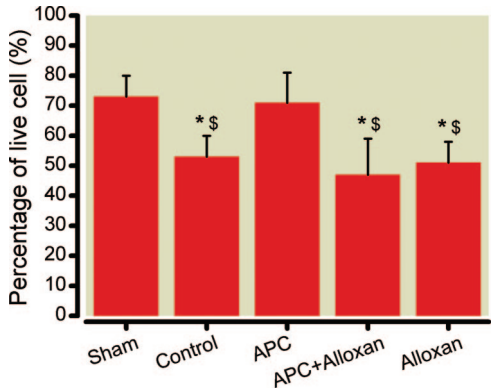

Fig. 4. Percentage of live cells after simulated ischemia-reperfusion. Anesthetic preconditioning (APC) prevented ischemia-reperfusion–induced injury of cardiac myocytes in the absence of alloxan. At least 200 cardiac myocytes were collected for each experiment (n = 6). * $P < 0.05$ *versus* APC. $ $P < 0.05$ *versus* sham. Mean ± SD, n = 6 per group.

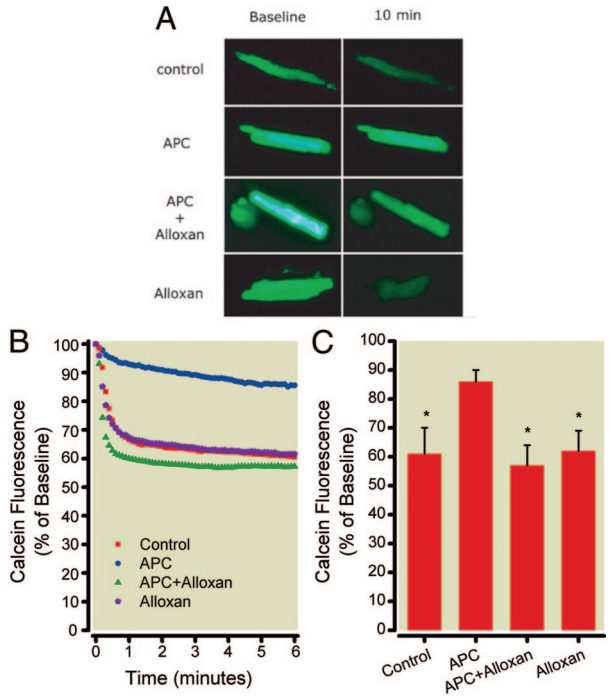

Fig. 5. Representative fluorescence images of calcein AM in mouse cardiac myocytes at baseline and 10 min after exposure to 100 μM H_2O_2 (oxidative stress) *(A)*. Time lapse (10 min) attenuation of fluorescence intensity of calcein AM in cardiac myocytes (n = 5), indicating oxidative stress (indicated by Calcein AM and cobalt chloride) induced the opening of the mitochondrial permeability transition pore (mPTP) *(B)*. Calcein AM fluorescence intensity of cardiac myocytes 10 min after exposure to 100 μM H_2O_2 shows that anesthetic preconditioning (APC) attenuated the opening of mPTP compared with sham-treated cardiac myocytes, but the effect was eliminated when cells were pretreated with alloxan. * $P <$ 0.05 *versus* APC. Mean ± SD, n = 5 per group *(C)*.

APC prevents opening of the mitochondrial permeability transition pore in response to oxidative stress in cardiac myocytes, but the protective effect is eliminated with alloxan treatment; and (5) isoflurane induces O-GlcNAc modification of VDAC. The results provide additional evidence that several proteins are modified by O-GlcNAc.[1] APC may induce O-GlcNAc posttranslational modification of proteins to result in cardiac protection.

Although the APC and IPC signaling pathways are similar, there are differences between the two.[17] Knowing the differences between these pathways may reveal new therapeutic targets for the prevention of myocardial infarction. O-GlcNAc is known to participate in the IPC signaling cascade, and the data herein suggests that O-GlcNAc signaling participates in APC.

Mitochondria are essential for cell survival and play important roles in the production of adenosine triphosphate and in the regulation of cell death. When the channel is open, mPTP allows low-molecular-weight molecules to enter the mitochondrial matrix, leading to an imbalance of osmotic pressure, matrix swelling, rupture of the outer mitochondrial membrane, and ultimately cell death. Ischemia-reperfusion injury induces calcium overload, and oxidative stress that, combined with other factors, leads to the formation of mPTP in the inner mitochondrial membrane.[18–20] It is also reported that the APC signaling pathway involves mPTP.[8] Three molecules are thought to be important structural components of mPTP, including adenine nucleotide translocase, cyclophilin D, and VDAC. Reports suggest that phosphorylation of VDAC by protein kinase A, protein kinase Cε, or glycogen synthase kinase 3β,[21–23] inhibits opening of mPTP, although this has been questioned.[24] In addition to phosphorylation, O-GlcNAc modification of VDAC may participate in the anesthetic cardioprotective signaling. Although there is still much to be learned about the function of O-GlcNAc–modified proteins in the heart, recent genetic studies have questioned whether VDAC is crucial[25] because the mPTP pore opens in fibroblasts that lack the three VDAC isoforms. However, strong evidence in favor of the importance of VDAC was obtained in heart mitochondria.[23] Taken together, the evidence both supports and refutes the involvement of VDAC in mPTP. Nevertheless, many O-GlcNAc–modified proteins are involved in cardiac protection, and VDAC probably plays a role in myocytes.

The current study demonstrated the O-GlcNAc modification of VDAC-1, a protein that functions not only in the formation of mPTP but also in adenosine triphosphate production and consumption. Anflous-Pharayra *et al.* reported that the function of VDAC-1 is related to multiple enzymatic activities within the respiratory chain that produce protons in the heart.[26] The responsible respiratory complex activities are different from those of VDAC-3, which participates only in complex IV. O-GlcNAc–modified VDAC-1 may be important for the production of adenosine triphosphate and when damaged may contribute to a lack of adenosine triphosphate and cardiac myocyte death.

The specific proteins modified by O-GlcNAc that mediate the protection against ischemia-reperfusion injury re-

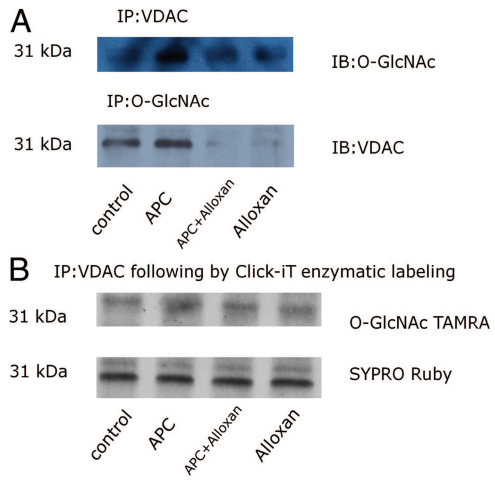

Fig. 6. Coimmunoprecipitation to detect O-linked β-*N*-acetylglucosamine (O-GlcNAc)–modified voltage-dependent anion channel (VDAC). Anesthetic preconditioning (APC) increased O-GlcNAc concentrations of the mitochondrial protein (VDAC) compared with control or alloxan alone. However, inhibition of O-GlcNAc transferase (OGT) with alloxan before APC reduced O-GlcNAc concentrations *(A)*. Immunoprecipitation for VDAC and enzymatic labeling of O-GlcNAc–modified proteins confirmed the result shown in figure 5A. SYPRO® Ruby staining showed there was no difference in total VDAC protein *(B)*. IB = immunoblot; IP = immunoprecipitation; TAMRA = tetramethylrhodamine-alkyne.

main to be identified. Kinases such as Akt, extracellular signal-regulated kinases 1 and 2, p38, and protein kinase C are known to play a role in mediating IPC,[27–28] and they are modified by O-GlcNAc or their activities are modulated by changes in O-GlcNAc concentrations.[2] Additional studies are warranted not only to identify cardiac proteins that are targets for O-GlcNAc modification but also to determine how these proteins are affected by ischemic stress and how changes in the concentrations of O-GlcNAc alter their response to stress.

Recently, mPTP inhibitors that interfere with opening of the channel have been used in clinical practice to reduce cardiac injury.[29] Development of novel specific inhibitors of mPTP or mitochondria-targeted reactive oxygen species scavengers may reduce mitochondria-mediated cardiac dysfunction in ischemic reperfusion. Additional investigations may target O-GlcNAc modifications to treat myocardial infarction or other metabolic diseases.

The limitation of this study is that we identified only one protein modified by O-GlcNAc. There may be other APC-signaling cascades related to O-GlcNAc–modified proteins. The other limitation is that alloxan has been used to inhibit O-GlcNAc transferase.[5,30–31] However, alloxan is a nonspecific inhibitor of OGT, and it was recently shown to inhibit β-D-*N*-acetylglucosaminidase,[32] the enzyme responsible for deglycosylation of O-GlcNAc–modified proteins. OGT and β-D-*N*-acetylglucosaminidase have opposite functions, but the result shown in figure 2 indicates that alloxan functioned as an inhibitor of OGT and not β-D-*N*-acetylglucosaminidase and that the O-GlcNAc modification of VDAC was eliminated by the addition of alloxan.

Volatile anesthetics increased O-glycosylation of cardiac proteins in mice. In addition, the O-glycosylation of the mitochondrial protein VDAC may inhibit opening of mPTP, which may explain the acquired resistance of cardiac myocytes to ischemic reperfusion injury.

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