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Volatile anesthetic post-treatment induces protection via inhibition of glycogen synthase kinase 3 β in human neuron-like cells

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Abstract

Application of the volatile anesthetic isoflurane during the early phase of reperfusion reduces ischemic heart and brain injury (anesthetic postconditioning). We hypothesize that inhibition of glycogen synthase kinase 3 β (GSK3 β), a protein whose activation can lead to cell death, participates in anesthetic postconditioning-induced neuroprotection. SH-SY5Y cells, a human neuroblastoma cell line, were induced by retinoic acid to differentiate into terminal neuron-like cells. The cells then were subjected to a 1-h oxygen-glucose deprivation (OGD), a condition to simulate ischemia *in vitro*, and a 20-h simulated reperfusion. Isoflurane, sevoflurane or desflurane, three commonly used volatile anesthetics, was applied for 1 h during the early phase of simulated reperfusion. Cell injury was quantified by lactate dehydrogenase (LDH) release. Phospho-GSK3 β at Ser9 and total GSK3 β were quantified at 1 or 3 h after the OGD. OGD increased LDH release, suggesting that OGD induced cell injury. Post-treatment with isoflurane, sevoflurane or desflurane reduced this cell injury. This protection was apparent when 2% isoflurane was applied within 1 h after the onset of reperfusion. Isoflurane post-treatment also significantly increased the phosphorylation of GSK3 β at Ser9 at 1 h after the OGD. GSK3 β inhibitors reduced OGD and simulated reperfusion-induced LDH release. The combination of GSK3 β inhibitors and isoflurane postconditioning did not cause a greater protection than isoflurane postconditioning alone. These results suggest that volatile anesthetic postconditioning reduces OGD and simulated reperfusion-induced cell injury. Since phospho-GSK3 β at Ser9 decreases GSK3 β activity, our results suggest that volatile anesthetic postconditioning in human neuron-like cells may be mediated by GSK3 β inhibition.

Keywords

glycogen synthase kinase 3 β ; human neuron-like cell; neuroprotection; postconditioning; volatile anesthetics

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Introduction

Ischemic postconditioning describes a phenomenon in which short episodes of interruptions of reperfusion reduce ischemic injury to various organs including brain and heart (Zhao et al., 2003; Zhao et al., 2006). This method of protection is considered to be very promising because its application to provide protection does not require prediction on when a detrimental ischemic event will occur. However, ischemic postconditioning, especially in the case of central nervous system, can be difficult to apply due to the requirement of delivering accurately controlled short episodes of ischemia. Thus, post-treatment or postconditioning with relatively safe agents will simplify the use of this protective modality and, thus, may have a broad application. We have shown that postconditioning with the volatile anesthetic isoflurane reduces ischemic brain injury in adult rats (Lee et al., 2008). A subsequent study showed that isoflurane post-treatment protected neonatal rat brain against hypoxia-ischemia (Zhou et al., 2010). It is not known whether other commonly used volatile anesthetics also can induce a postconditioning effect in the brain. The mechanisms for volatile anesthetic postconditioning-induced neuroprotection are largely unknown.

It has been shown that activation of pro-survival signaling molecules, such as protein kinase B (PKB)/Akt, is involved in ischemic postconditioning-induced organ protection (Zhao, 2009). PKB/Akt can phosphorylate glycogen synthase kinase 3 β (GSK3 β) (Matsui and Rosenzweig, 2005; Zhao, 2009). Phosphorylation of GSK3 β at Ser9 inhibits GSK3 β (Isagawa et al., 2000; Gosens et al., 2007), which then reduces opening of the mitochondrial permeability transition pore (mPTP) (Juhászova et al., 2004; Gomez et al., 2008; Juhászova et al., 2008). It is known that mPTP opening induces cell death (Petronilli et al., 2001; Korde et al., 2007), an important mechanism for ischemic brain injury. Thus, we hypothesize that post-treatment with commonly used volatile anesthetics induces neuroprotection and that this protection requires inhibition of GSK3 β . To test these hypotheses, we induced the SH-SY5Y cells to differentiate into terminal neuron-like cells. Oxygen-glucose deprivation (OGD) was used to simulate ischemia *in vitro*.

Materials and Methods

Cell culture

SH-SY5Y cells, a human neuroblastoma cell line, were obtained from the American Type Culture Collection (Manassas, VA) and cultured as we described before (Zuo et al., 2006). Briefly, the cells were cultured in a T75 flask containing 13 ml of Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 nutrient mixture (1:1) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were kept at 37°C in a humidified incubator gassed with 95% air and 5% CO₂. The medium was changed twice per week. When the cells were 70 – 80% confluent, they were exposed to 0.25% trypsin-EDTA solution and sub-cultured in a new flask.

The SH-SY5Y cells were plated in 6-well plates for lactate dehydrogenase (LDH) release assay at a density of 1 $\times$ 10⁵ cells/cm² or 100-mm dishes for Western blotting at a density of 1 $\times$ 10⁶ cells/cm². One day after plating, cells were incubated in neurobasal medium, supplemented with B-27 supplement (GIBCO, Carlsbad, CA) and L-glutamine (500 μ M; Nacalai Tesque Inc., San Diego, CA). Retinoic acid (RA, 10 μ M; Sigma, St Louis, MO) was added to the medium for 3 days to induce SH-SY5Y cells to differentiate into a homogenous population of cells with neuronal morphology (Kume et al., 2008; Lin et al., 2011). These cells then were used in the experiments.

Oxygen-glucose deprivation

Cells in control group were washed with phosphate buffered saline (PBS) and incubated in neurobasal medium in a humidified atmosphere of 95% air and 5% CO₂ at 37 C. The exposure of cells to OGD was performed as we described before (Kim et al., 2009b; Wu et al., 2010). Briefly, neurobasal-A medium that did not contain L-glucose (GIBCO) was bubbled with 100% N₂ for 30 min. Cells first were washed with PBS once and 2 ml/well of the neurobasal-A medium was added to the cells. These plates were immediately placed in an air-tight chamber (Billups-Rothenberg, Inc., Del Mar, CA) gassed with 100% N₂ for 10 min. The oxygen content in the outlet of the chamber was monitored with a DatexTM infrared analyzer (Capnomac, Helsinki, Finland) and reached 2% at 3 – 5 min after the onset of gassing. After closure of the inlet and outlet of the chamber, the chamber was kept at 37 C for 1 h except for the time-course experiment in which the OGD exposure times were 1, 3, 5 and 10 h. After confirming that the oxygen content in the chamber was still lower than 2% at the end of the OGD period, the chamber was opened and glucose, B-27 supplement and L-glutamine (500 μM) was added to make the final glucose concentration in the buffer at 4.5 g/l. The plates then were kept for 20 h in a humidified atmosphere of 95% air and 5% CO₂ at 37 C.

Anesthetic exposure

The cells were placed in an airtight chamber immediately after OGD. The chamber then was gassed with 1%, 2% or 3% isoflurane or sevoflurane or 3%, 6% or 9% desflurane in the carrying gases (95% air and 5% CO₂) for 15 min. The volatile anesthetic concentrations in the gases from the outlet of the chamber were monitored with a DatexTM infrared analyzer and reached the target concentrations at ~3 min after the onset of gassing. The chamber was sealed and the incubation was for 1 h at 37°C. At the end of incubation, the anesthetic concentrations in the gases from the chamber were confirmed to be at the target concentrations by the infrared analyzer. The plates then were kept for 19 h in a humidified atmosphere of 95% air and 5% CO₂ at 37 C. In another experiment, 2% isoflurane was applied for 1 h at 0, 1, 2, 4, 6, 8 or 16 h after the OGD. As we reported previously (Xu et al., 2008; Kim et al., 2009a), the aqueous isoflurane concentrations at 37°C were ~209, 415 or 620 μM, respectively, as measured by gas chromatography when 1, 2 or 3% isoflurane was delivered and the liquid samples were taken for measurements at the end of the 1-h isoflurane exposure.

Recently, relatively long-exposures to volatile anesthetics have been shown to cause cell injury (Xie et al., 2007). However, we have showed that exposure of the differentiated SH-SY5Y cells to 2 – 4% isoflurane, 6% sevoflurane or 12% desflurane for 48 h does not cause a significant injury or change in expression of synaptic proteins in these cells (Lin, et al., 2011). Thus, the anesthetic exposure conditions used in this study were not expected by themselves to cause significant injury to the differentiated SH-SY5Y cells.

Application of glycogen synthase kinase 3β inhibitors

To determine the concentration-response of GSK-3β inhibitors on OGD-induced injury of the differentiated SH-SY5Y cells, the highly selective GSK-3β inhibitors Chir 98014 or Chir 99021 (Axon Medchem BV, Groninger, Netherlands) (Ring et al., 2003) were added to the cells at the onset of OGD to make the final concentrations at 10, 100, 150 and 200 nM for Chir 98014 and 50, 200, 500 and 1000 nM for Chir 99021 in the incubation solution. In another experiment, 150 nM Chir 98014 or 500 nM Chir 99021 was added at the onset of OGD. These cells then were exposed to 2% isoflurane for 1 h immediately after the OGD.

Lactate dehydrogenase (LDH) activity assay

LDH activity was determined using an LDH cytotoxicity detection kit as we did before (Kim, et al., 2009a; Kim, et al., 2009b). Briefly, the incubation solution harvested at the end of experiments was centrifuged at 13,000 rpm for 10 min. One hundred micro-liters of the supernatant were transferred to 96-well plates and incubated with the same volume of reaction mixture from the kit. The samples then were read in a spectrophotometry (Bio-Rad Laboratories, Hercules, CA) with the absorbance wavelength at 492 nm and the reference wavelength at 655 nm. Background absorbance from the cell-free buffer solution was subtracted from all absorbance measurements. After removal of the incubation solution from 6-well plates, 1% triton X-100 lysing solution was applied to each well to dissolve the remaining cells. The percentage of LDH released to incubation buffer in total LDH was calculated as follows: LDH in the buffer / (LDH in the buffer + intracellular LDH released by triton X-100).

Western blotting

The differentiated SH-SY5Y cells in 100 mm dishes were subjected to or not subjected to 1-h OGD. They then were exposed to or not exposed to 2% isoflurane for 1 h. Cells were harvested at 1 h or 3 h after the OGD and homogenized in a lysing buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 30 μ M MG132 and protease inhibitor mixture (complete; Roche Applied Science, Indianapolis, IN). Homogenates were centrifuged at 4 C for 30 min at 13,000 rpm. Protein content in the supernatant was determined using a Bio-Rad Protein Assay Kit. Thirty micrograms of proteins per lane were loaded and electrophoresed in a 10% polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% w/v bovine serum albumin and 0.1% Tween-20 in PBS and then incubated with the following primary antibodies: rabbit polyclonal anti-phospho-GSK3 β (Ser9) antibody (1:1000, catalogue number: 9336, Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-GSK3 β antibody (1:1000, catalogue number: 9315, Cell Signaling Technology) and rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000, catalogue number: G9545, Sigma chemical, St Louis, MO). Appropriate secondary antibodies were used and protein bands were visualized using a Genomic and Proteomic Gel Documentation (Gel Doc) Systems from Syngene (Frederick, MD). The protein band intensities of phospho-GSK3 β and total GSK-3 β were normalized by the corresponding band intensities of GAPDH from the same samples. The results from cells under various experimental conditions then were normalized by those of the corresponding control cells.

Statistical analysis

Data are expressed as means $\pm$ S.D. ($n \geq 12$ for each experimental condition). Results were analyzed by one-way analysis of variance followed by the Tukey test for *post hoc* analysis after confirmation of normal distribution of the data or by Kruskal-Wallis analysis of variance on ranks followed by the Dunn's test when the data are not normally distributed. A *P* value < 0.05 was considered statistically significant. All statistical analyses were performed with the SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

Results

The human neuron-like cells differentiated from the SH-SY5Y cells responded to OGD and simulated reperfusion with an increase of LDH release to culture medium. This increase of LDH release was OGD length-dependent. The degree of LDH release in the control cells was not changed over time (Fig. 1). These results suggest that these neuron-like cells were injured by OGD. Since 1-h OGD induced a significant increase of LDH release compared

with the corresponding control, we chose to use this condition for the subsequent experiments.

Exposure to 1, 2 or 3% isoflurane for 1 h immediately after OGD significantly reduced the OGD and simulated reperfusion-induced LDH release. However, exposure to 2% isoflurane for 1 h no longer significantly reduced the OGD-induced LDH release if the isoflurane exposure did not occur within 1 h after the OGD (Fig. 2). Similarly, exposure to the newer volatile anesthetics sevoflurane or desflurane for 1 h immediately after OGD also significantly reduced the OGD-induced LDH release (Fig. 3).

Although OGD and isoflurane did not cause a significant change of the total GSK3 β expression in the differentiated SH-SY5Y cells harvested at 1 h or 3 h after the OGD, both of them significantly increased the phosphorylation of GSK3 β at Ser9. Of note, the OGD plus isoflurane condition caused a greater increase in the Ser9 phosphorylation than OGD alone at 1 h after the OGD (Fig. 4).

Chir 98014 and Chir 99021, two very selective GSK3 β inhibitors (Ring, et al., 2003), dose-dependently decreased OGD-induced LDH release. The EC₅₀ for this effect was 9.3 and 49.7 nM, respectively, for Chir 98014 and Chir 99021 (Fig. 5). Since 150 nM Chir 98014 and 500 nM Chir 99021 had maximized this effect (Fig. 5), they were used in the subsequent experiments. Interestingly, the combination of these GSK3 β inhibitors and the exposure of cells to 2% isoflurane caused a greater reduction of the OGD-induced LDH release than the GSK3 β inhibitors alone. However, the inhibition of the OGD-induced LDH release caused by the combination of GSK3 β inhibitors and isoflurane exposure was not different from that induced by isoflurane exposure alone (Fig. 6).

Discussion

We have shown that postconditioning with isoflurane reduces ischemia-induced brain injury in rats (Lee, et al., 2008). We now show that isoflurane also induces a postconditioning effect in human neuron-like cells. Delayed application of isoflurane up to 1 h after OGD still induces protection. Consistent with this finding, our previous study showed that isoflurane application within 20 min after the onset of simulated reperfusion provided rat brain slices with protection against OGD and simulated reperfusion (Lee, et al., 2008). These results suggest that there is a short post-OGD/ischemia time window within which isoflurane application can induce neuroprotection.

In addition to isoflurane, we show here that the newer volatile anesthetics desflurane and sevoflurane also induce a postconditioning effect. This induction occurs at clinically relevant concentrations of these three anesthetics. These results suggest that induction of a postconditioning effect in neural tissues may be a common feature of volatile anesthetics. Consistent with this suggestion, it has been shown that sevoflurane also induces a postconditioning effect in rabbit spinal cord (Wang et al., 2009).

One of the popular theories to explain ischemic postconditioning-induced protection is the “pH hypothesis” (Cohen et al., 2007). The short episodes of ischemia or low pressure reperfusion at the early phase of reperfusion would maintain tissue acidosis that will provide the ischemic tissues with time needed to activate reperfusion injury salvage kinases, such as PKB/Akt, before the occurrence of reperfusion injury (Gross and Auchampach, 2007; Zhao, 2009). Unlike in the case of rapid reperfusion that induces the production of a large amount of free radicals from tissues, attenuated reperfusion may produce a low level of free radicals (Gross and Auchampach, 2007). While a large amount of free radicals is detrimental, a small amount of free radicals can activate pro-survival signaling molecules, such as PKB/Akt (Gross and Auchampach, 2007; Zhao, 2009).

A key molecule that is downstream of PKB/Akt for producing protective effects is GSK3 β . PKB/Akt can phosphorylate GSK3 β at Ser9 (Matsui and Rosenzweig, 2005; Zhao, 2009). This phosphorylation inhibits GSK3 β , (Isagawa, et al., 2000; Gosens, et al., 2007) which then can decrease mPTP opening (Juhászova, et al., 2004; Gomez, et al., 2008; Juhászova, et al., 2008). Although detailed mechanisms for GSK3 β inhibition to lead to decrease of the mPTP opening are not known, it is proposed that inhibition of GSK3 β reduces the formation of mPTP (Miura et al., 2009). In contrast, it is well-established that mPTP opening allows release of many molecules, including cytochrome C, from the mitochondria (Petronilli, et al., 2001; Korde, et al., 2007). This process is a key event to induce cell apoptosis (Li et al., 2008). mPTP opening also has been shown to induce cell necrosis (Nakagawa et al., 2005). Thus, GSK3 β has been considered as a master switch kinase for cell survival. Consistent with this idea, multiple studies have shown a critical role of GSK3 β inhibition in cardioprotection induced by various agents and modalities (Gomez, et al., 2008; Juhászova et al., 2009). Inhibition of GSK3 β or phosphorylation of GSK3 β at Ser9 has been indicated to mediate isoflurane postconditioning-induced cardioprotection (Feng et al., 2005).

Limited information suggests that GSK3 β also may play a role in determining brain cell fate after detrimental insults (Petit-Paitel et al., 2009). For example, lithium reduces ischemia-induced neuronal death, which may be mediated by inhibition of GSK3 β via increasing GSK3 β phosphorylation at Ser9 (Bhat et al., 2000; Sasaki et al., 2006). However, it is not known yet whether GSK3 β inhibition contributes to a postconditioning effect in the brain. Our results showed that OGD alone significantly increased the phosphorylation of GSK3 β at Ser 9, suggesting that OGD may induce an endogenous response to reduce cell injury. Consistent with our findings, previous studies have shown that the expression of phospho-GSK3 β at Ser 9 in rat brains is increased in the early phase (hours) after focal brain ischemia (Sasaki, et al., 2006; Gao et al., 2008). We also showed that the addition of isoflurane to OGD caused a further increase of phosphorylation of GSK3 β at Ser9 at 1 h after the OGD. In addition, Chir 98014 and Chir 99021 dose-dependently reduced OGD and simulated reperfusion-induced cell injury.

These two agents are among the most selective GSK3 β inhibitors. Their IC₅₀s are at low nanomolar or sub-nanomolar levels to inhibit GSK3 β and at high micromolar levels to inhibit other protein kinases, such as PKB/Akt (Ring, et al., 2003). Our study showed that the EC₅₀ was 9.3 and 49.7 nM, respectively, for Chir 98014 and Chir 99021 to inhibit the OGD and simulated reperfusion-induced cell injury. These EC₅₀s are consistent with their affinities for GSK3 β reported previously (Ring, et al., 2003). Finally, the combination of Chir 98014 and isoflurane or Chir 99021 and isoflurane did not induce a better protective effect than isoflurane alone. These three lines of evidence strongly suggest that the inhibition/phosphorylation of GSK3 β contributes to the isoflurane-induced postconditioning effects in human neuron-like cells.

We used Chir 98014 and Chir 99021 at doses that maximized their protective effects in the study to assess the effects of the combination of these inhibitors with 2% isoflurane (Fig. 5). Two percent isoflurane also maximized isoflurane protective effect (Fig. 2). Our results showed that the protection induced by the combination of agents was similar to that induced by isoflurane alone but greater than the GSK3 β inhibitor alone. These results suggest that GSK3 β inhibition contributes to the isoflurane postconditioning effects and that additional mechanism(s) may play a role in the isoflurane effects. Consistent with this suggestion, we previously have shown a role of the mitochondrial ATP sensitive potassium channels in the isoflurane-induced postconditioning effects in rat brain (Lee, et al., 2008).

There are limitations in our study. We differentiated the human SH-SY5Y cells into neuron-like cells because it is almost impossible to obtain human neural tissues or neurons to

perform the experiments. Obviously, there are significant differences between our neuron-like cells and human neurons *in situ*. Thus, one has to be cautious to extrapolate our results directly to human situation. Also, we included GSK3 β inhibitors in the incubation solution for 21 h (from the onset of OGD to the end of experiments). Ideally, the inhibitors should be present only during the period when GSK3 β is inhibited by isoflurane to imply the role of GSK3 β inhibition in the isoflurane-induced protection. However, it is not practical to change the incubation solution to remove the GSK3 β inhibitors in the middle of experiments. In addition, the results that GSK3 β inhibitors did not induce a greater protection than isoflurane alone suggest that the long incubation of cells with GSK3 β inhibitors may not have introduced significant confounding issues in interpreting our results.

In summary, we showed that isoflurane, sevoflurane and desflurane, three currently used volatile anesthetics, induced a postconditioning effect in human neuron-like cells. The application of volatile anesthetics needed to occur in the early phase of reperfusion (within 1 h) in order to induce this protection. Inhibition of GSK3 β at least partly contributes to this volatile anesthetic postconditioning effect.

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Abbreviations

DMEM	Dulbecco's modified Eagle medium
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase 3 β
LDH	lactate dehydrogenase
mPTP	mitochondrial permeability transition pore
OGD	oxygen-glucose deprivation
PKB	protein kinase B

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Fig. 1. Time course of oxygen-glucose deprivation (OGD)-induced cell injury

The differentiated human SH-SY5Y cells were subjected to various lengths of OGD followed with a 20-h simulated reperfusion. Cell injury was assessed by lactate dehydrogenase (LDH) release. Results are means $\pm$ S.D. (n = 24). * P < 0.05 compared with the corresponding time control cells, ^ P < 0.05 compared with cells subjected to 1-h OGD and then a 20-h simulated reperfusion.

Fig. 2. Protective effects of isoflurane post-treatment

(A) Dose-response of isoflurane effects on oxygen-glucose deprivation (OGD)-induced cell injury. The differentiated human SH-SY5Y cells were subjected to 1-h OGD followed with a 20-h simulated reperfusion. Cells were exposed to 1, 2, or 3% isoflurane for 1 h immediately after the onset of simulated reperfusion. Results are mean $\pm$ S.D. ($n = 24$). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD only. **(B)** Time-window of delayed isoflurane post-treatment. The differentiated human SH-SY5Y cells were subjected to 1-h OGD followed with a 20-h simulated reperfusion. Cells were exposed to 2% isoflurane for 1 h at 0, 1, 2, 4, 6, 8 and 16 h after the initiation of the simulated reperfusion. Results are mean $\pm$ S.D. ($n = 36 - 48$). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD only.

Fig. 3. Dose-response of the effects of sevoflurane or desflurane post-treatment on oxygen-glucose deprivation (OGD)-induced cell injury

The differentiated human SH-SY5Y cells were subjected to 1-h OGD followed with a 20-h simulated reperfusion. Cells were exposed to various concentrations of sevoflurane or desflurane for 1 h immediately after the onset of simulated reperfusion. Results are mean $\pm$ S.D. (n = 24). * P < 0.05 compared to control. ^ P < 0.05 compared to OGD only.

Fig. 4. Effects of isoflurane post-treatment and oxygen-glucose deprivation (OGD) on glycogen synthase kinase 3 β (GSK3 β) expression in human neuron-like cells

The differentiated human SH-SY5Y cells were subjected to or not subjected to 1-h OGD followed with or without the exposure to 2% isoflurane for 1 h. They then were harvested at 1 h (panel A) or 3 h (panel B) after the OGD for Western blotting. Results are mean $\pm$ S.D. (n = 12). * P < 0.05 compared to control. ^ P < 0.05 compared with OGD only. p-GSK3 β : phospho-GSK3 β .

Fig. 5. Dose-response of the effects of glycogen synthase kinase 3 β (GSK3 β) inhibitors on oxygen-glucose deprivation (OGD)-induced cell injury

The differentiated human SH-SY5Y cells were subjected to 1-h OGD followed with a 20-h simulated reperfusion. Various concentrations of GSK3 β inhibitors (Chir 98014 and Chir 99021) were added to cells just before the OGD. Results are mean $\pm$ S.D. (n = 24). * P < 0.05 compared to control. ^ P < 0.05 compared to OGD only.

Fig. 6. Interaction between the protections induced by isoflurane post-treatment and glycogen synthase kinase 3β (GSK3β) inhibitors

The differentiated human SH-SY5Y cells were subjected to 1-h OGD followed with a 20-h simulated reperfusion. GSK3β inhibitors (150 nM Chir 98014 or 500 nM Chir 99021) were added to cells just before the OGD. Cells were exposed to or not exposed to 2% isoflurane for 1 h immediately after the onset of OGD. Results are mean $\pm$ S.D. (n = 24). * P < 0.05 compared to control. ^ P < 0.05 compared to OGD only. # P < 0.05 compared with OGD plus Chir 98014 (98014). \$ P < 0.05 compared with OGD plus Chir 99021 (99021).