Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia

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Shintani-Ishida K, Uemura K, Yoshida K. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 293: H1714–H1720, 2007.—The aim of this study was to investigate changes in hemichannel activity during in vitro simulated ischemia [oxygen-glucose deprivation (OGD)] and the contribution of hemichannels to ischemia-reperfusion injury in rat neonatal cardiomyocytes. Dye uptake assays showed that hemichannels opened as OGD progressed, peaking after 1 h, and then closed, returning to the pre-OGD state after 2 h of OGD. The increase in dye uptake after 1 h of OGD was inhibited by hemichannel blockers (lanthanum chloride and a connexin 43 mimetic peptide, Gap26). During OGD, intracellular Ca2+ concentration ([Ca2+]i) began to increase after 1 h and reached several micromolar after 2 h. After 1 h of OGD, Gap26 inhibited the increases in hemichannel activity and [Ca2+]i. In contrast, dantrolene [an endo(sarco)plasmic reticulum Ca2+ release inhibitor] suppressed the increase in [Ca2+]i, but not in hemichannel activity. After 2 h of OGD, the combined administration of 2,4-dichlorobenzamil and dantrolene reduced [Ca2+]i to <1 μM and increased hemichannel activity to the level attained after 1 h of OGD. Simulated ischemia-reperfusion, induced by 1 h of OGD followed by 2 h of recovery, reduced cell viability to 54% of the control level. The addition of Gap26 to OGD medium improved viability to 80% of the control level. In conclusion, this study demonstrated that 1) hemichannels open transiently during OGD, 2) closure of hemichannels, but not their opening, is regulated by an increase in [Ca2+]i, during OGD, and 3) open hemichannels contribute to cell injury during recovery from OGD.

gap junction; connexin; ischemia-reperfusion

HEMICANNELS EXIST IN PLASMA membranes and are precursors to gap junctions (28); each gap junction channel is formed by the docking of two hemichannels, one from each of two neighboring cells (12, 23). In cardiomyocytes, hemichannels are composed of connexin proteins (4). Hemichannels are known to close in static conditions and to open in response to membrane depolarization (9, 38), mechanical stress (3), extracellular Ca2+ loss (14), and high pH (39). Kondo et al. (20) and John et al. (19) demonstrated that functional connexin hemichannels exist in isolated rabbit ventricular myocytes and are opened by metabolic inhibition. Additionally, it has recently been reported that in vitro simulated ischemia [oxygen-glucose deprivation (OGD)] results in the opening of neuronal hemichannels, which are composed of connexin 43 (37). Pannexins belong to a family distinct from the connexins (6). It therefore remains to be addressed whether simulated ischemia opens connexin hemichannels in cardiomyocytes.

Open hemichannels act as nonselective conduits for cations and small molecules, allowing the release of ATP (43) and Na+ (7) as well as the influx of extracellular Na+ and Ca2+ (22). Ischemia rapidly disturbs ionic homeostasis and induces an increase in intracellular Ca2+ concentration ([Ca2+]i), resulting in cellular injury and death (31). These findings suggest that hemichannels contribute to cell injury due to ischemia-reperfusion. Consistent with this hypothesis, it has been shown that metabolic inhibition opens hemichannels (19) and that the hemichannel blocker lanthanum chloride (La3+) delays the onset of cell injury due to metabolic inhibition (8). However, whether hemichannels are involved in ischemia-reperfusion injury is not known.

De Vuyst et al. (10) found that a moderate increase in [Ca2+]i triggers hemichannel opening, whereas an excessive increase in [Ca2+]i causes them to close. These findings prompted us to speculate that hemichannels open or close during ischemia depending on [Ca2+]i and contribute to cardiomyocyte injury due to ischemia-reperfusion.

The aim of this study was thus to investigate hemichannel activity in relation to [Ca2+]i during ischemia and the contribution of hemichannels to ischemia-reperfusion injury in rat neonatal cardiomyocytes.

MATERIALS AND METHODS

Primary culture of rat myocytes and experimental design. Primary cardiomyocyte cultures were prepared from ventricles of 1-day-old Sprague-Dawley rats as described previously (35) and grown at 37°C for 5–6 days before use in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 Nutrient Mixture (Sigma-Aldrich, St. Louis, MO) containing 5% FBS and 1× Insulin-Transferrin-Selenium-X (Invitrogen, Carlsbad, CA). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Tokyo. For simulated ischemia, cells were exposed to OGD medium, i.e., Hanks’ balanced salt solution (in mM: 125 NaCl, 4.9 KCl, 1.2 MgSO4, 8.0 NaHCO3, and 20 HEPES, pH 6.4) free of serum, glucose, and oxygen (gassed with N2 for 15 min) for 15–120 min.

Dye uptake. Dye uptake assay was performed with the hemichannel-permeable reporter dye ethidium bromide (Eth) as described previously (8). Cells cultured in 96-well plates were incubated with 100 μM Eth in OGD medium for 2 min at the end of the period of OGD and washed twice with PBS. This incubation period resulted in little background labeling. For visualization of Eth uptake in Fig. 1, A–J, images (2 positions for each well) were acquired using a TE2000-E inverted microscope in epifluorescence mode (tetramethylrhodamine isothiocyanate excitation/emission) with a charge-coupled device camera (CoolSNAP, Nippon Roper, Tokyo, Japan). For quantification of Eth dye uptake in Figs. 1K, 2, 3, and 6, dye

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fluorescence was measured with excitation at 535 nm and emission at 595 nm using a plate reader (GENios, Tecan, Männedorf, Switzerland). In some experiments, a connexin 43 mimetic peptide, Gap26 (0.25 mg/ml) (5), was present in the medium throughout OGD and dye application. Lanthanum chloride (La3+ (0.25 mg/ml) (5), was present in the medium throughout OGD, and fluorescence was measured with excitation at 340 and 380 nm for quantification of dye uptake with Lucifer yellow (LY, 100 μM; Fig. 1I), dextran-fluorescein (Dex-FITC, 100 μM; Fig. 1J), and dextran-fluorescein (Dex-FITC, 100 μM; Fig. 1J). Cells were incubated in OGD medium (●) or normal culture medium (control, ○) for 15–120 min before dye addition. Each value (mean ± SD, n = 8) represents net difference in fluorescence between 0 min and each time point and is derived from 1 of 3 independent experiments with interexperiment adjustment. *P < 0.05 vs. 0 min.

Intracellular Ca2+ measurement. Cell cultures in black 96-well plates were loaded with fura 2 by ester loading. Before OGD, cells were incubated in culture medium containing 5 μM fura 2-AM (Dojindo Laboratories, Kumamoto, Japan) at 37°C for 60 min and washed twice with culture medium without fura 2-AM. To prevent dye leakage, 1.25 mM probenecid (Wako, Osaka, Japan) was included in the medium during dye loading. For measurement of fura 2 fluorescence, cells were washed once with OGD medium at the end of OGD, and fluorescence was measured with excitation at 340 and 380 nm and emission at 520 nm using a plate reader (GENios). In some experiments, Gap26 (0.25 mg/ml), verapamil (1 μM), mibefradil (10 μM), efonidipine (10 μM), KB-R7943 (10 μM), 2,4-dichlorobenzamid (10 μM), or dantrolene (100 μM) was applied throughout dye loading for 60 min and OGD.

[Ca2+]i was calculated as described by Grynkiewicz et al. (15) according to the following formula: [Ca2+]i = Kd × β × (R - Rmin)/(Rmax - R), where Kd is the dissociation constant of fura 2 for Ca2+, Rmin and Rmax are the 340- to 380-nm fluorescence ratios at zero and saturating[Ca2+]i, respectively, and β is the ratio of fluorescence at 380 nm at zero [Ca2+]i to that at saturating [Ca2+]i. To establish the Kd value in our experiments, we performed a preliminary in vivo calibration in cells using calibration solutions containing 1 μM ionomycin with different [Ca2+]i as described previously (40).

Cell viability. Cell viability was assessed using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer’s instructions. Briefly, cells cultured in 96-well plates were subjected to 1 h of OGD followed by 2 h of recovery in culture medium (phenol red free). After simulated ischemia-reperfusion (OGD recovery), highly water-soluble disulfonated tetrazolium salt (WST-8) solution (10 μl, final concentration 0.5 mM) was added to each well, and the cells were incubated for ~30 min. WST-8 (colorless) changes to WST-8 formazan (orange) through the action of intracellular dehydrogenases (18). The formazan dye was measured at 460 nm using a plate reader (GENios). Gap26 or dantrolene was added to the medium 1 h before OGD, kept constant throughout OGD, and removed before recovery.

Statistical analysis. Data were analyzed using two-way ANOVA and the Tukey-Kramer test at a confidence level of 95%. All determinations, including dye uptake, [Ca2+]i, measurement, and cell viability assay, were performed in parallel to minimize variation due to daily and lot-to-lot reagent variations, and the results of each experiment are expressed as values normalized with respect to controls.

RESULTS

Hemichannels open transiently during OGD. Hemichannels are permeable to Eth (charge +1) (13). In rat neonatal cardiomyocytes in control medium, cells were not labeled during incubation with Eth for 2 min (Fig. 1A). Many cells were labeled when Eth was applied for 2 min at the end of 60 min
of OGD (Fig. 1B). Eth labeling was seen as fluorescence of the nuclei (Fig. 1, J vs. I). Few labeled cells were observed after 120 min of OGD (Fig. 1C). To quantitatively investigate the temporal change in hemichannel activity during OGD, Eth uptake was determined from full-field measurements of fluorescence using a plate reader. Cells were subjected to OGD for 15, 30, 60, 90, or 120 min and incubated with Eth for 2 min at the end of each OGD period (Fig. 1K). Consistent with the images of Eth-labeled cells (Fig. 1, A–C), Eth uptake increased with the duration of OGD, peaking at 60 min, and returned to control levels by 120 min. Hemichannels are also permeable to LY (charge −2) (13). Application of LY for 2 min showed a similar temporal change in hemichannel activity during OGD (Fig. 1L). In contrast, cells were not labeled with Dex-FITC (molecular mass 10 kDa) during OGD (Fig. 1, E–G), fluorescence measurements using a plate reader did not detect Dex-FITC uptake during OGD (Fig. 1M). These results indicate that cells transiently take up small, but not large, molecules during OGD through a process that does not involve membrane disruption.

We confirmed that 1 h of OGD increases Eth uptake through hemichannels using hemichannel and gap junction blockers (Fig. 2). Extracellular La3+ is known to block hemichannel currents (100 μM) (37) and dye uptake through hemichannels (50 μM) (8). Gap26 (0.25 mg/ml), a peptide that corresponds in sequence to a short segment of the first extracellular loop of connexin 43, is a specific blocker of hemichannels formed by connexin 43. The addition of La3+ or Gap26 blocked the increase in Eth uptake after 1 h of OGD. Another peptide, sGap (0.25 mg/ml), which has a scrambled sequence of composition without glucose deprivation, Eth uptake was at control levels.

Fig. 2. Effects of hemichannel and gap junction blockers on Eth uptake after 1 h of OGD. Cells were incubated in modified OGD medium (gray bars) without glucose supplementation, instead of OGD medium (solid bar). As control (C), cells were incubated in normal culture medium (open bar). Values (means ± SE, n = 8) represent fluorescence relative control. *P < 0.05.

These results indicate that the transient increase in Eth uptake during OGD is specifically mediated by hemichannels.

The simulated ischemia is induced by hypoxia (N2 gas bubbling), acidic pH (6.4), and glucose deprivation. Accordingly, we investigated which of these conditions contributes to hemichannel opening during OGD. We compared the effect of omitting each condition individually during the preparation of OGD medium on Eth uptake (Fig. 3). Eth uptake levels were similar in hypoxic and nonhypoxic conditions. In contrast, Eth uptake was reduced at neutral pH or when glucose supplementation was provided. Additionally, under neutral conditions without glucose deprivation, Eth uptake was at control levels.

Hemichannel opening induces an increase in [Ca2+]. We monitored [Ca2+]i during OGD (Fig. 4). A steep increase in [Ca2+]i started after 1 h of OGD and reached ~3 μM after 2 h of OGD.

We investigated whether hemichannel opening after 1 h of OGD induced the increase in [Ca2+]. The hemichannel blocker Gap26 suppressed the increase in [Ca2+]i after 1 h of OGD (Fig. 5A). Dantrolene [a known blocker of Ca2+ release
Hemichannels are involved in cell injury on recovery from OGD. Simulated ischemia-reperfusion (OGD recovery) induces cell injury and the death of myocardial cells (30, 34). These findings led us to examine the contribution of hemichannels to cell injury during OGD recovery.

First, we examined the effect of the duration of OGD on cell viability after OGD recovery (Fig. 7). Cells were exposed to OGD for 15, 30, 60, 90, or 120 min and allowed to recover in culture medium for 2 h before determination of cell viability. Cell viability decreased with the duration of OGD, declining to 54% of the control level after 1 h of OGD.

Second, we examined whether hemichannels remained open during recovery after 1 h of OGD. Cells were allowed to recover for 0, 15, 30, 60, or 120 min after 1 h of OGD and then subjected to dye uptake assay for 2 min (Fig. 8). To distinguish Eth uptake through hemichannels from that resulting from membrane disruption during OGD recovery, cells were incubated simultaneously with Eth and Dex-FITC. The numbers of cells labeled with Eth, but not with Dex-FITC, were increased during 0–30 min of recovery after 1 h of OGD (Fig. 8, D and E) compared with control cells. These results indicate that hemichannels are maintained in an open state during recovery (for ≥30 min) after OGD.

Third, to investigate whether open hemichannels are involved in cell injury during OGD recovery, we examined the mechanism of cell injury (Fig. 9). Cells were exposed to OGD for 1 h and then allowed to recover for 0, 15, 30, 60, or 120 min before addition of 2,4-dichlorobenzamil and dantrolene (Fig. 5B). A combination of these two reagents increased Eth uptake to the level attained after 1 h of OGD (Fig. 6). These results are consistent with the observation that the combined, but not the single, administration of these reagents reduced [Ca\(^{2+}\)]\(_i\) to <1 μM (Fig. 5B). The increase in Eth uptake resulting from the combined administration of the two reagents was inhibited by the addition of Gap26 (Fig. 6). These results support our hypothesis that hemichannels close when [Ca\(^{2+}\)]\(_i\) surpasses a threshold for hemichannel closure during OGD.

In summary, we conclude that hemichannels are involved in cell injury after ischemia-reperfusion (OGD recovery). Simulated ischemia-reperfusion (OGD recovery) induces cell injury and hemichannel opening. A combination of 2,4-dichlorobenzamil and dantrolene reduced Eth uptake from that resulting from membrane disruption during OGD recovery, suggesting that hemichannels remain open during recovery after OGD.

Fig. 5. Effects of hemichannel, Ca\(^{2+}\) channel, and Na\(^+\)/Ca\(^{2+}\) exchanger blockers and endoplasmic reticulum Ca\(^{2+}\) release inhibition on increase of [Ca\(^{2+}\)]\(_i\) without OGD (open bars) and after 1 h (A) and 2 h (B) of OGD (solid bars). Values are means ± SE (n = 8). Non, no inhibitor; Gap, Gap26; Ve, verapamil; Mi, mibefradil; Ef, efonidipine; KB, KB-R7943; Di, 2,4-dichlorobenzamil; Da, dantrolene. *P < 0.05 vs. OGD without inhibitor. **P < 0.001 vs. each non-OGD group (A) and vs. OGD without inhibitor (B).

Fig. 6. Effect of [Ca\(^{2+}\)]\(_i\), on hemichannel activity after 1 and 2 h of OGD. Cells were subjected to OGD for 1 or 2 h before dye application (solid bars). In non-OGD groups (open bars), cells were incubated with culture medium, instead of OGD medium. Values (means ± SE, n = 8) represent fluorescence relative to that in non-OGD group for each inhibitor. Non, no inhibitor; Da, dantrolene; Di, 2,4-dichlorobenzamil; C, combined administration of 2,4-dichlorobenzamil and dantrolene; C + Gap, combined administration of 2,4-dichlorobenzamil, dantrolene, and Gap26. *P < 0.05 vs. non-OGD.
effect of Gap26 on cell viability after 2 h of recovery from 1 h of OGD (Fig. 9). When Gap26 was added to OGD medium, cell viability during recovery from OGD was increased to 80% of the control level. In contrast, a Gap26 negative control (sGap) did not increase cell viability. Dantrolene inhibited the elevation in \([\text{Ca}^{2+}]_i\) to an extent similar to that caused by hemichannel blockers after 1 h of OGD (Fig. 5A) but did not prevent cell injury during recovery from OGD (Fig. 9). These results indicate that hemichannels are involved in cell injury during recovery from OGD independently of \([\text{Ca}^{2+}]_i\) overloading.

**DISCUSSION**

This is the first report on the transient opening of hemichannels in rat cardiomyocytes during simulated ischemia (OGD) and the contribution of hemichannels to cell injury due to simulated ischemia-reperfusion (OGD recovery). Consistent with our findings, it was recently reported that OGD causes neuronal hemichannels in mouse brain slices and isolated hippocampus neurons to open (37). We demonstrated not only hemichannel opening but also the subsequent closure of hemichannels during OGD. Hemichannels opened with increasing duration of OGD, peaking at 1 h, and then closed when \([\text{Ca}^{2+}]_i\) reached a threshold level, returning to the pre-OGD state after 2 h of OGD.

A moderate increase in \([\text{Ca}^{2+}]_i\) has been reported to trigger hemichannel opening and an excess of \([\text{Ca}^{2+}]_i\) to cause their closure (10). However, in cardiomyocytes subjected to OGD, it is unlikely that hemichannel opening is regulated by an increase in \([\text{Ca}^{2+}]_i\). We found that hemichannels were maximally open after 1 h of OGD, just as \([\text{Ca}^{2+}]_i\) began to increase (Figs. 1 and 4). However, dantrolene inhibited the increase in \([\text{Ca}^{2+}]_i\) (Fig. 5A), but not in hemichannel activity (as demonstrated using dye uptake; Fig. 6).

We found that acidic pH and glucose deprivation partially contribute to the opening of hemichannels during OGD (Fig. 3). Since it has been reported that acidic pH (intracellular or extracellular) causes hemichannels to close (32, 39), it is unlikely that acidic pH is directly responsible for the opening of hemichannels during OGD. Hemichannels are known also to open in response to membrane depolarization (9, 14, 38), which can be induced by ischemia (1). This possibility remains to be addressed.

The hemichannel blocker Gap26, as well as dantrolene, blocked the increase in \([\text{Ca}^{2+}]_i\) after 1 h of OGD (Fig. 5A).
Unexpectedly, the Na\(^{+}/\)Ca\(^{2+}\) exchange inhibitors 2,4-dichlorobenzamil (27, 36) and KB-R7943 (21) enhanced the increase in [Ca\(^{2+}\)]\(_i\) (Fig. 5A). Our data suggest that Ca\(^{2+}\) influx is enhanced through hemichannels and that the increased intracellular Ca\(^{2+}\) is extruded through Na\(^+\)/Ca\(^{2+}\) exchangers in early ischemia (1 h of OGD). Ca\(^{2+}\) loading is known to be sensitive to the beating rate in cardiomyocytes. We found that the beating rate was increased with KB-R7943, 2,4-dichlorobenzamil, and dantrolene but decreased with verapamil, mibebradil, and efondipine in control and OGD medium (data not shown). However, the change in beating rate induced by each inhibitor cannot explain changes in [Ca\(^{2+}\)]. Although a limitation of inhibitors on the specificity remains, the consistency of the data obtained with different inhibitors supports our conclusions.

Between 1 and 2 h of OGD, the Ca\(^{2+}\) influx pathway appears to differ from that in early ischemia. The increase in [Ca\(^{2+}\)], after 2 h of OGD was not inhibited by the hemichannel blocker Gap26 (Fig. 5B), consistent with low hemichannel activity (Fig. 1). Ca\(^{2+}\) influx is likely to be partly promoted by Ca\(^{2+}\) regulators, such as L-type (16) or T-type (26, 29) Ca\(^{2+}\) channels, Na\(^+\)/Ca\(^{2+}\) exchangers (27, 36), and endo(sarco)plasmic reticulum Ca\(^{2+}\) release channels (42), as demonstrated by the small effect of inhibitors of each (Fig. 5B). These results may indicate that hemichannels mediate Ca\(^{2+}\) loading only in early ischemia.

The combined administration of 2,4-dichlorobenzamil and dantrolene, but not their separate use, maintained a high level of hemichannel activity after 2 h, comparable to that after 1 h. In addition, the hemichannel blocker Gap26 countered the maintenance of hemichannel activity by these two inhibitors after 2 h of OGD (Fig. 6). It is noteworthy that the combined, but not the separate, administration of these two reagents also reduced [Ca\(^{2+}\)]\(_i\) to <1 μM (Fig. 5B), which would maintain hemichannels in an open state. Consistent with our results, De Vuyst et al. (10) reported that an increase in [Ca\(^{2+}\)]\(_i\) to >1 μM results in hemichannel closure. Collectively, these results suggest that hemichannels close at a threshold of >1 μM [Ca\(^{2+}\)]\(_i\) during 1–2 h of OGD and that the open state is maintained when [Ca\(^{2+}\)]\(_i\) is kept below the threshold level by the combined use of 2,4-dichlorobenzamil and dantrolene.

The involvement of hemichannels in cell injury due to simulated ischemia-reperfusion was also demonstrated for the first time in the present study. We found that 1 h of OGD followed by 2 h of recovery in culture medium maintains hemichannels in an open state (Fig. 8) and reduces cell viability (Fig. 9). Although [Ca\(^{2+}\)]\(_i\) elevation after 1 h of OGD was reduced by dantrolene and Gap26 (Fig. 5A), cell viability after recovery from OGD was improved by Gap26, but not by dantrolene (Fig. 9). These results suggest that hemichannels promote cell injury independently of [Ca\(^{2+}\)]\(_i\) elevation after 1 h of OGD. Hemichannels mediate not only Ca\(^{2+}\) influx but also Na\(^+\) influx (22) and NAD\(^{+}\) (7) and ATP release (43). The contribution of each of these to reperfusion injury remains to be addressed.

Li et al. (24) reported that connexin 43 contributes to a cardioprotective effect of ischemic preconditioning not through gap junction intracellular communication but, rather, through regulation of cellular volume in isolated cardiomyocytes without gap junctions. This finding suggests an involvement of hemichannels in ischemic preconditioning. Hemichannels would be involved in not only ischemia-reperfusion injury but, also, ischemic preconditioning’s protection.

In conclusion, hemichannels open transiently during simulated ischemia (OGD), thereby promoting cell injury during recovery from OGD in rat neonatal cardiomyocytes. The closure of hemichannels, but not their opening, is regulated by increased [Ca\(^{2+}\)]\(_i\) during OGD. The interaction between hemichannel activity and Ca\(^{2+}\) regulation has been elucidated in ischemia.

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REFERENCES

HEMICHANNELS TRANSIENTLY OPEN DURING ISCHEMIA


