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Volume-sensitive outwardly rectifying chloride channels are involved in oxidative stress-induced apoptosis of mesangial cells

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Abstract

Volume-sensitive outwardly rectifying (VSOR) Cl^- channels have been electrophysiologically identified in human and mouse mesangial cells, but the functional role of VSOR Cl^- channels in mesangial cell apoptosis is not clear. The aim of the present study was to demonstrate the role of VSOR Cl^- channels in oxidative stress-induced mesangial cell apoptosis. H_2O_2-induced Cl^- currents showed phenotypic properties of VSOR Cl^- channels, including outward rectification, voltage-dependent inactivation at more positive potentials, sensitivity to hyperosmolarity, and inhibition by VSOR Cl^- channel blockers. Moreover, blockage of VSOR Cl^- channels by DIDS (100 μM), NPPB (10 μM) or niflumic acid (10 μM) rescued mesangial cell apoptosis induced by H_2O_2. Treatment with 150 μM H_2O_2 for 2 h resulted in significant reduction of cell volume, in contrast, nuclear condensation and/or fragmentation were not observed and the caspase-3 activity was also not increased. The early-phase alterations in cell volume were markedly abolished by pretreatment with VSOR Cl^- channel blockers. We conclude that VSOR Cl^-channels are involved in H_2O_2-induced apoptosis in cultured mesangial cells and its mechanism is associated with apoptotic volume decrease processes.

Keywords: Apoptosis; Mesangial cells; Volume-sensitive chloride channels; Apoptotic volume decrease; Ion channels; Glomerulosclerosis; Kidney disease

In renal glomerulus, apoptosis of mesangial cells has been observed in both experimental and human glomerular diseases [1–5]. Some studies implicated apoptosis of mesangial cells as the major mechanism for the resolution of glomerular hypercellularity in experimental proliferative glomerulonephritis [1,2]. Whereas others suggested that its uncontrolled activation was involved in the glomerular cell loss in progressive glomerulosclerosis [3,4]. These data indicated that mesangial cell apoptosis was an important event in the pathogenesis of glomerulosclerosis. Oxidative stress is thought to be a trigger of mesangial cell apoptosis. Mesangial cells and infiltrating leukocytes are capable of generating reactive oxygen species (ROS) in response to proinflammatory stimuli [6]. Moreover, a large body of evidence suggested that ROS, such as hydrogen peroxide (H_2O_2), superoxide anion, and peroxynitrite, initiated mesangial cell apoptosis in vitro [7–9]. Multiple signaling pathways involved in oxidative stress-induced apoptosis of mesangial cells have been identified, including c-Jun N-terminal kinase (JNK)–activator protein 1 (AP-1) and extracellular signal regulated kinase (ERK)–AP-1 pathways [8,9]. However, the mechanisms of mesangial cell apoptosis induced by oxidative stress are not completely understood.

Recently, the concept that volume-sensitive outwardly rectifying (VSOR) Cl^- channels expressed in plasma membrane are involved in apoptotic cell death in response to a variety of stimuli has been proposed [10–12]. In tumor cells, T lymphocytes, and cardiomyocytes, VSOR Cl^- currents were activated by death receptor- or mitochondria-mediated apoptotic stimulation, and its inhibition by

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VSOR Cl^- channel blockers, such as diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), or niflumic acid, led to rescuing these cells from apoptotic death [13–16]. Given that VSOR Cl^- channels play an important role in the regulation of cell volume, it is presumed that the mechanisms, in which VSOR Cl^- channels participate in cell apoptosis, are associated with the induction of apoptotic volume decrease (AVD) [10,12,17]. Normotonic shrinkage of cells during the apoptosis processes, termed AVD, is a major hallmark and early event of programmed cell death, preceding cytochrome *c* release, caspase-3 activation, and DNA fragmentation [12,17]. The induction of AVD is coupled with the facilitation of regulatory volume decrease (RVD) [13], a main regulation mechanism of cells in response to hypotonic stress, indicating that apoptosis is accompanied with disordered volume regulation. Bortner et al. [18] reported that persistent shrinkage induced by hyperosmotic stress led to apoptotic death in lymphocytes. AVD-initiated decrease in intracellular ion may also affect the activation of apoptotic proteins [19]. For example, processing of proIL-1β to its mature form by the interleukin β converting enzyme (ICE), also known as caspase-1, occurred at low intracellular potassium levels in human monocytes [20], whereas exposure of apoptotic-stimulated cells to high extracellular potassium resulted in the inhibition of apoptosis [21]. The relationship between AVD-initiated decrease in intracellular Cl^- ion and activation of caspase has not been completely understood, but some studies showed that inhibition of AVD by VSOR Cl^- channel blockers prevented the activation of caspase-3 induced by tumor necrosis factor-α (TNF-α) or staurosporine (STS) [13]. These data indicated that AVD was an early prerequisite to apoptotic cell death.

These findings provided a new clue for revealing the mechanism of mesangial cell apoptosis triggered by oxidative stress. In human and mouse mesangial cells, VSOR Cl^- channels have been identified [22], but their role in oxidative stress-induced apoptosis is not clear. Therefore, the present study aimed at elucidating the role of VSOR Cl^- channels in H_2O_2-induced apoptosis in cultured mesangial cells, subsequently, the possible mechanism of this phenomenon through evaluating the changes of cell volume.

Materials and methods

Reagents. RPMI 1640, fetal bovine serum, and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). DIDS, NPPB, niflumic acid, and other electrophysiological reagents were purchased from Sigma (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit was obtained from Roche company (Penzberg, Germany). CaspACE assay system, fluorometric kit, was purchased from Promega (WI, USA). Other reagents if not indicated were purchased from Sigma (St. Louis, MO, USA).

Cell culture. An established stable human mesangial cell line (HMCL) was used in all experiments (kindly donated by Dr. J.D. Sraer, Hopital Tenon, Paris) [23]. These cells were cultured in RPMI 1640 medium supplemented with 10% FCS and incubated at 37 °C in a humidified atmosphere (95% air–5% CO_2). For cell apoptosis measurements, cells were cultured in 10% FCS overnight and serum-deprived (1% FCS) for 24 h. Then, the cells were pretreated with or without VSOR channel inhibitors: DIDS (100 μM), NPPB (10 μM), and niflumic acid (10 μM) for 2 h and exposed to H_2O_2 (150 μM) for 12 h. For all experiments cells in passages 51–65 were used.

Whole cell patch clamp recording. Electrophysiological experiments were performed as described previously [24]. Mesangial cells were seeded on pieces of glass coverslips at a density of 1×10^5/ml overnight. The coverslips were placed on a recording chamber, which were mounted on an inverted microscope (Diaph-TMD, Nikon, Tokyo, Japan). The solution flowing over the cells per minute was ≈10 times greater than the volume of the chamber. Patch pipettes were pulled from borosilicate thin-wall glass capillaries (TW150F-4, World Precision Instruments, Sarasota, FL) with a micropipette puller (P-97, Sutter Instrument, Novato, CA) and had tip resistances of 3–7 MΩ when filled with electrode solution. The pipette solution was composed of (in mM): 106 *N*-methyl-D-glucamine chloride (NMDG-Cl), 10 Hepes, 1 EGTA, and 5 Mg-ATP, adjusted to pH 7.1 with Tris base. Osmolarity of the pipette solution was tested with a vapor pressure osmometer (5120C, Wescor, Logan, UT) and adjusted to 290 mOsm with mannitol. The bath solution contained (in mM) 100 NMDG-Cl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 5 Tris base, 10 glucose, and adjusted to pH 7.4. Osmolarity of the solution was adjusted to 290 mOsm with mannitol, equal to that of the pipette solution.

The membrane currents were recorded in whole cell configuration with an Axopatch 200B amplifier (Axon Instruments, Redwood City, CA). Data were acquired at 10 kHz and filtered at 2 kHz. Cell capacitance and series resistance (Rs) compensation was performed with the Axopatch amplifier. Rs was compensated up to 80%. As the Cl^- concentration of the bath solution was equal to that of the pipette solution, the junction potential between bath and pipette solution was less than 1 mV, therefore, it was ignored. All experiments were performed at room temperature (20–23 °C).

Fluorescent microscopy measurements. Mesangial cells were seeded on a glass coverslip. After the induction of apoptosis, the coverslips were treated with 20μl of freshly prepared acridine orange/ethidium bromide (AO/EB) solution (100 μg/ml AO and 100 μg/ml EB in PBS) and then were viewed under a fluorescent microscope (Olympus, Tokyo, Japan). Cell images were captured by a CCD digital camera (CoolSNAP cf, Roper Scientific, Arizona, USA). Apoptosis was identified using morphological criteria, including nuclear condensation and/or fragmentation.

Assessment of electron microscopy. To perform electron microscopy analysis, mesangial cells were grown in 25-cm^2 culture flasks. After induction of apoptosis, these cells were fixed in 2.5% glutaraldehyde in PBS for 2 h at 4 °C and then postfixed in 1% osmium tetroxide. After dehydration in a series of graded ethanol baths (30–100%) and then in propylene oxide, cells were embedded in Epon. Cell sections (80–200 nm) were obtained using a Reichert Ultracut E microtome and stained with uranyl acetate. Grids were examined with a Jeol 1200 EXII electron microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. Mesangial cells were gown on a glass overslip. After induction of apoptosis, these cells were subjected to TUNEL assays according to the manufacturer's protocol. In brief, cells were fixed by 4% paraformaldehyde for 1 h at 4 °C. The samples were permeabilized with 0.2% Triton X-100 for 2 min at 4 °C. After washing with PBS, the samples were incubated with reaction mixture for 1 h at 37 °C and then were analyzed by fluorescence microscopy (Olympus, Tokyo, Japan).

Cell volume measurements. Mesangial cells were grown on 10-mm petri dishes. After induction of apoptosis, the dishes were mounted on an inverted microscope (Olympus, Tokyo, Japan) with a CCD camera. Cell images were captured every 5 min throughout the entire experiments and then was analyzed with an image analysis software (Image-Pro Plus 5.02, Memia Cybernetics, MD, USA). The relative cell volume changes (V/V_0) were calculated from the cross-sectional surface area at the beginning (S_0) of the experiments and during (S) the experiments from the relation: $V/V_0 = (S/S_0)^{3/2}$. This approach has been previously found to be very much correlated to cell volume changes in renal A6 cells [25], lymphocytes [26], and cardiac myocytes [16]. All experiments were performed at room temperature (20–23 °C).

Caspase-3 activity assays. The caspase-3 activity was assayed using CaspACETM Assay System, fluorometric Kit (Promega, WI, USA), according to the instructions provided by the manufacturer. Briefly, cells were lysed and the supernatant were used for measurements of caspase-3 activity. The fluorogenic substrates for caspase-3 were labeled with the fluorochrome 7-amino-methyl coumarin (AMC). AMC was released from these substrates upon cleavage by caspase-3. The enzyme activity was determined by monitoring the fluorescence produced by free AMC using a fluorometer (Hitachi High Technologies, F-4500, Wokingham, UK) at 360/460 nm. Assays were performed in the presence and absence of selective inhibitors for caspase-3 to assess the specific contribution of this enzyme. Caspase-3 activity was expressed in picomoles AMC liberated/minute/microgram protein.

Statistical analysis. Data were expressed as means ± SE. Statistical analysis was performed using unpaired Student's *t* test to compare data in different groups. p value <0.05 was used to indicate a statistically significant difference.

Results

H_2O_2 activates VSOR Cl^- currents

To measure Cl^- currents, conditions were designed so that cation currents were minimized. In these experiments, NMDG was used to replace intracellular K^+ and extracellular Na^+ minimizing the cation channel currents. Intracellular and extracellular Cl^- was symmetrical at 106 mM, therefore, Cl^- reversal potential or equilibrium potential for Cl^- (E_{Cl}) was 0 mV under this condition. Command potentials were held at 0 mV and stepped up to various potentials. As shown in Figs. 1A and B, treatment with 150 μM H_2O_2 elicited activation of a fast activated, outwardly rectifying current. At positive potentials the currents showed a slightly voltage-dependent inactivation. The current density was increased from -0.40 ± 0.04 and 0.97 ± 0.21 to -3.53 ± 0.57 and 8.77 ± 0.32 pA/pF at -80 and $+100$ mV, respectively ($p < 0.01$, $n = 15$). The whole cell currents reversed close to the theoretical E_{Cl} (0 mV) at 0.3 ± 0.5 mV ($n = 15$). Furthermore, replacing extracellular Cl^- with equimolar gluconate shifted the reversal potentials to $+17.8 \pm 0.6$ mV ($n = 3$). The current magnitude was markedly reduced and the outwardly rectification was lost. These data suggested that the currents induced by H_2O_2 were predominantly carried by Cl^-. The properties of H_2O_2-induced Cl^- currents were similar

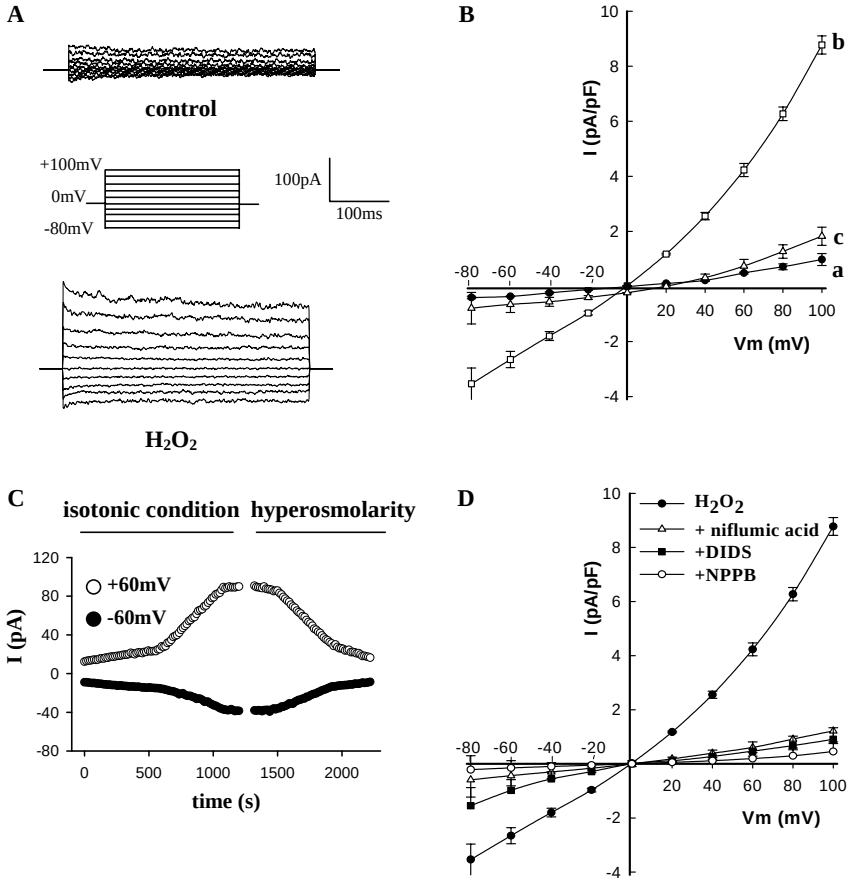

Fig. 1. H_2O_2 activates VSOR Cl^- channels. (A) Current traces recorded from a representative mesangial cell under control (top) and in the presence of 150 μM H_2O_2 (bottom). The voltage protocol for current recordings is shown in the inset. (B) Current–voltage relations under control conditions (trace a) and in the presence of 150 μM H_2O_2 (trace b) ($n = 15$). However, replacing extracellular Cl^- with equimolar gluconate shifted the reversal potentials to a more positive value (trace c) ($n = 3$). (C) Time course of H_2O_2-activated currents from a representative cell at +60 mV (open symbols) and −60 mV (filled symbols). Data were obtained from the voltage ramp protocol, which was applied every 15 s. (D). Current–voltage relations for H_2O_2-induced Cl^- currents in the absence or presence of VSOR Cl^- channel blockers, DIDS (100 μM), or NPPB (10 μM) or niflumic acid (10 μM). Protocol is same to (A), $n = 3$.

to those of VSOR chloride currents in many cell types in respect to its outward rectification and voltage-dependent inactivation [36]. We further examined its inhibition by hyperosmolarity. Indeed, application of hypertonic bath solution (120% osmolarity) significantly suppressed the Cl^- currents as shown in Fig. 1C. We next determined the effects of known VSOR chloride channel blockers on H_2O_2-induced Cl^- currents. As shown in Fig. 1D, both of NPPB (10 μM) and niflumic acid (10 μM) caused voltage-independent inhibition of the H_2O_2-induced Cl^- current ($95 \pm 12\%$ and $85 \pm 12\%$ at $+80$ mV, respectively, $n = 3$), while DIDS resulted in a voltage-dependent inhibition ($56 \pm 17\%$ and $89 \pm 14\%$ at -80 and $+80$ mV, respectively, $n = 3$). These results indicate that H_2O_2-induced activation of VSOR chloride channels under isotonic conditions.

H_2O_2-induced apoptosis is prevented by VSOR Cl^- channel blockers

To determine the role of VSOR Cl^- channels in mesangial cell apoptosis induced by H_2O_2, cells were pretreated with or without DIDS (100 μM), NPPB (10 μM) or niflumic acid (10 μM) and exposed to 150 μM H_2O_2. As shown in Figs. 2A and B, exposure of mesangial cells to H_2O_2 resulted in morphological alterations characteristic of apoptosis, such as nuclear condensation and fragmentation. However, these morphological signs of apoptosis were largely abolished by pretreatment with DIDS as shown in Fig. 2C. Other VSOR Cl^- channel inhibitors, NPPB or niflumic acid, were also effective (Figs. 2D and E). These effects were further confirmed using transmission electron microscopy. Ultrastructural changes

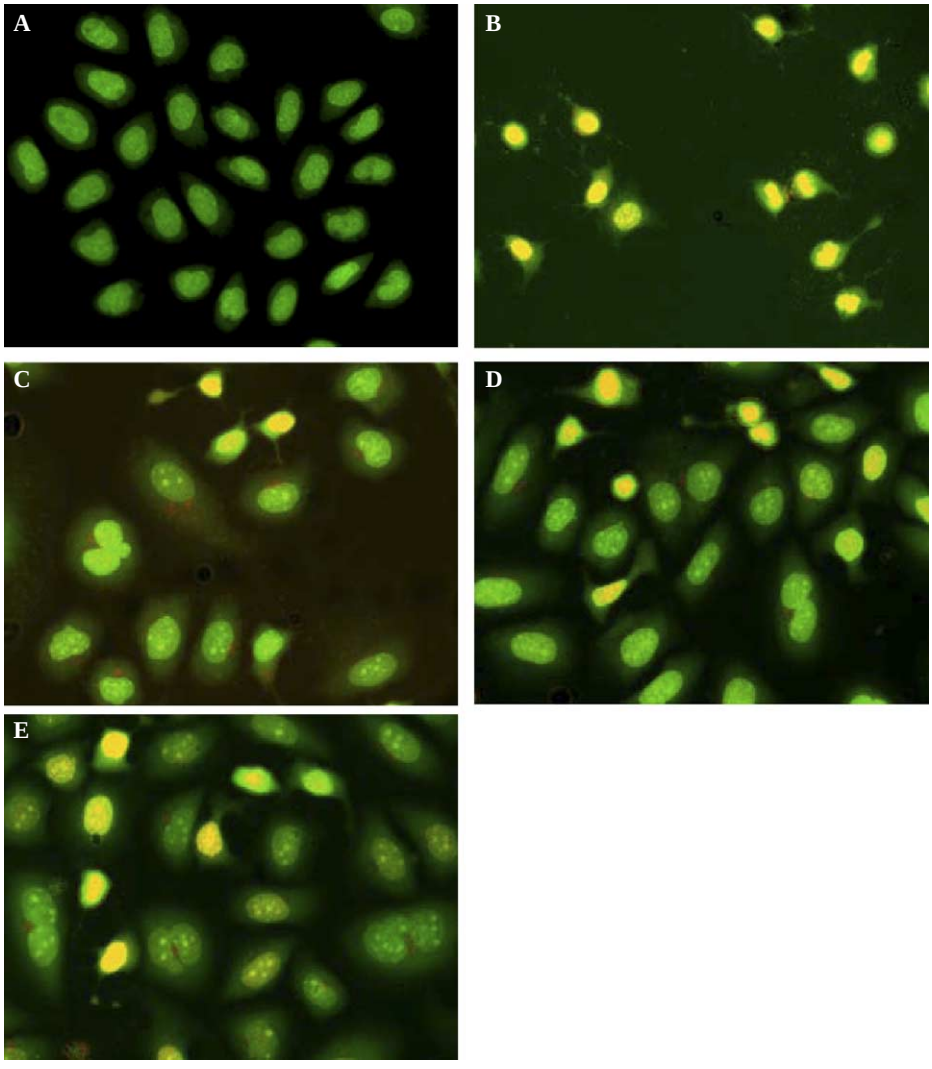

Fig. 2. Assessment of apoptosis by AO/EB staining. Mesangial cells cultured in 1% FCS for 24 h were pretreated with or without DIDS (100 μM), or NPPB (10 μM) or niflumic acid (10 μM) and then were exposed to H_2O_2 (150 μM) for 12 h. (A) Control cells showed no signs of apoptosis. (B) Treatment with 150 μM H_2O_2 resulted in typically apoptotic events featuring with a high number of cells with condensed and/or fragmented nuclei. Pretreatment with DIDS (C) or NPPB (D) or niflumic acid (E) significantly abolished the morphologically typical changes of apoptosis. Assays were performed in quadruplicate. 200 ×Magnification.

typical of apoptosis, including chromatin margination and nuclear condensation, induced by 150 μM H_2O_2 were markedly attenuated by the presence of DIDS, NPPB or niflumic acid (Fig. 3).

Consistent with the results of morphological analysis, quantitative analysis showed that the percentage of apoptosis was markedly increased in H_2O_2-exposed cells (Fig. 4). When the cells were pretreated with 50 or 100 μM DIDS and then exposed to H_2O_2, the number of TUNEL-positive cells was reduced to $12 \pm 2.6\%$ and $34 \pm 3.1\%$, respectively, suggesting that the inhibitory effect of DIDS on apoptosis induced by H_2O_2 is dose-dependent. This effect was also observed by pretreatment with other VSOR Cl^- channel inhibitors, NPPB or niflumic acid.

The effects of VSOR Cl^- channel inhibitors on caspase-3 activation triggered by H_2O_2 were also examined. When cells cultured in 1% FCS for 24 h were exposed to 150 μM H_2O_2 for 6 h, the caspase-3 activity markedly increased (Fig. 5). However, the increase in caspase-3 activity triggered by H_2O_2 was abolished by pretreatment of DIDS, NPPB or niflumic acid (Fig. 5).

Taken together, these results indicated that pharmacological blockage of VSOR Cl^- channels led to inhibiting H_2O_2-induced apoptosis in cultured mesangial cells.

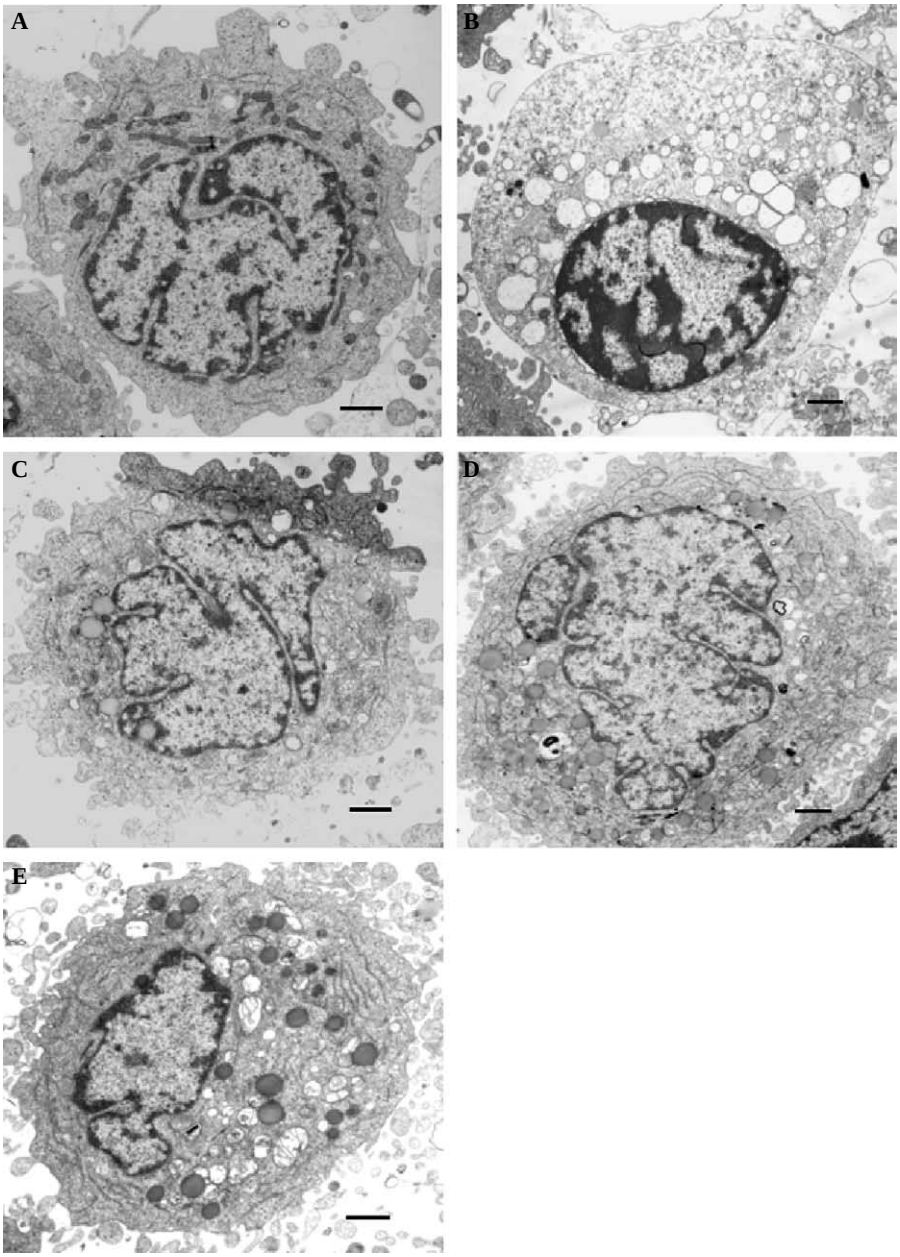

Fig. 3. Assessment of apoptosis by transmission electron microscopy. In control cells (A), ultrastructurally typical alterations of apoptosis were not visible. H_2O_2-exposed cells (B) exhibited chromatin condensation and margination, and the morphological features were markedly attenuated by pretreatment with DIDS (C), NPPB (D) or niflumic acid (E).

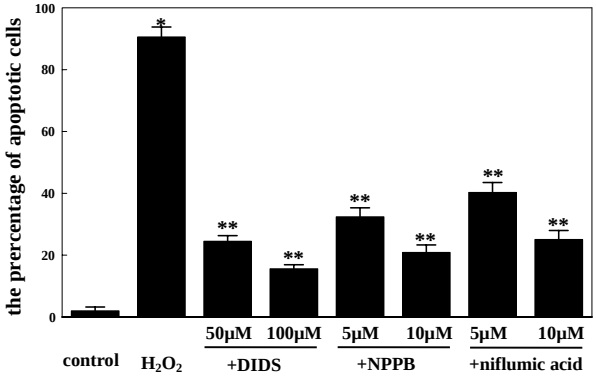

Fig. 4. Quantitative analysis of apoptosis by TUNEL staining. Mesangial cells cultured in 1% FCS for 24 h were exposed to 150 μM H_2O_2 in the presence or absence of DIDS (50, 100 μM), NPPB (5, 10 μM), and niflumic acid (5, 10 μM) for 12 h, and then subjected to TUNEL staining. *$p < 0.01$, versus control, **$p < 0.01$, versus H_2O_2-exposed cells, $n = 6$.

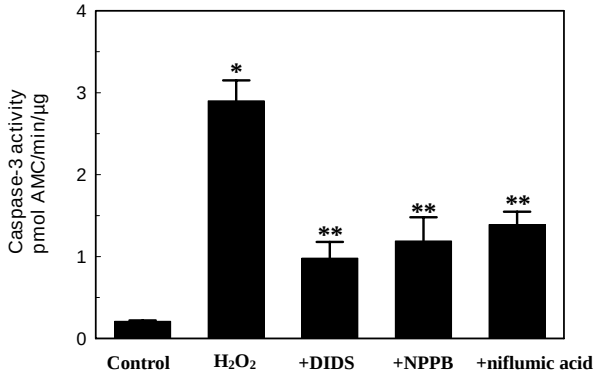

Fig. 5. The effects of VSOR Cl^- channel inhibitors on caspase-3 activation triggered by H_2O_2. Mesangial cells cultured in 1% FCS for 24 h were exposed to 150 μM H_2O_2 in the presence or absence of DIDS (100 μM), NPPB (10 μM), and niflumic acid (10 μM) for 12 h, and then the caspase-3 activity was detected. *$p < 0.01$, versus control, **$p < 0.01$, versus H_2O_2-exposed cells, $n = 6$.

Blockage of VSOR Cl^- channels abrogates H_2O_2-induced AVD

We examined whether H_2O_2-induced AVD was regulated by VSOR Cl^- channels in mesangial cells. Treatment with 150 μM H_2O_2 for 2 h resulted in significant reduction in cell volume (Fig. 6A), but nuclear condensation and/or fragmentation were not observed using AO/EB staining (data no shown) and the caspase-3 activity was not increased (Fig. 6B). However, the early-phase alterations in cell volume were markedly abolished with pretreatment with DIDS, NPPB or niflumic acid. These data suggested that VSOR chloride channels are involved in AVD progress induced by H_2O_2.

Discussion

Oxidative stress plays a key role in induction of mesangial cell apoptosis, an important pathologic event for progression of and recovery from glomerular injury [1–4].

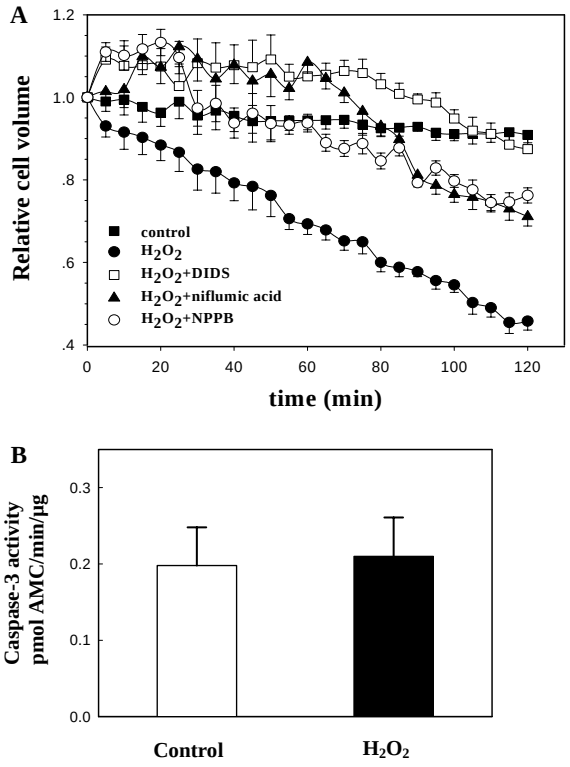

Fig. 6. The effects of VSOR Cl^- channel inhibitors on AVD induced by H_2O_2. Mesangial cells cultured in 1% FCS for 24 h were exposed to 150 μM H_2O_2 in the presence or absence of DIDS (100 μM), NPPB (10 μM), and niflumic acid (10 μM) for 2 h. (A) The time course of cell volume changes during 2 h (in 32 cells from 6 experiments). (B) The caspase-3 activity changes within 2 h. There was no significant difference between control and H_2O_2-exposed cell groups. $p > 0.05$, $n = 6$.

However, the precise molecular mechanisms are currently not well understood. Ion channels are involved not only in events leading to contraction of mesangial cells, but also have a role in the mechanisms leading to cell proliferation [27]. However, little information is available on the role of ion channels in mesangial cell apoptosis. This study demonstrated that VSOR Cl^- channels participate in H_2O_2-induced apoptosis and the possible mechanisms may be related to their induction of AVD. This was based on the facts that; (i) VSOR Cl^- channels were activated by apoptosis inducer, H_2O_2, and pharmacologic blockade of these channels suppressed H_2O_2-induced apoptosis. (ii) Blockers of these channels prevented AVD processes and activation of caspase-3.

As have been reported in other cells [14,28], H_2O_2-induced Cl^- currents in mesangial cells showed phenotypical properties of VSOR Cl^- channels, including outward rectification, voltage-dependent inactivation at more positive potentials, inhibition by classic Cl^- blockers, and sensitivity to hyperosmolarity. Moreover, pharmacological blockade of VSOR Cl^- channels led to rescuing these cells from apoptotic cell death, implying that activation of VSOR Cl^- currents is not only involved in oxidative stress-induced apoptosis, but also is required. The link between VSOR Cl^- channels and apoptosis also has been revealed in

Xenopus oocytes, in which proapoptotic stimuli activated the endogenous VSOR Cl^- currents [29]. In some cell types, such as ECV403 cells [30] and U937 [13], apoptotic cell death was abolished by phloretin, which, at a concentration below 100 μM, blocks VSOR Cl^- channels but not cAMP- or Ca^{2+}-activated Cl^- channels [31]. These data further suggested the involvement of VSOR Cl^- channels in apoptosis.

Accumulating evidence suggested that H_2O_2-induced apoptosis is mainly through mitochondrion-mediated pathways in mesangial cells [32]. In fact, it has been suggested that in many cell types, VSOR Cl^- channels participate not only in mitochondrion-mediated apoptosis, but also in death receptor-mediated apoptosis. For example, in Jurkat T lymphocytes and HeLa cells, proapoptotic stimuli, such as ceramide, tumor necrosis factor (TNF), or anti-Fas antibody, also activated VSOR Cl^- channels and their pharmacological blockade antagonized apoptosis[14,15]. These data implied that activation of VSOR Cl^- channels is a common characteristic of cell apoptosis and thereby has a key role in regulation of apoptotic events.

An important issue is how VSOR Cl^- channels are activated during apoptosis induced by a variety of proapoptotic stimuli. Some signal transducing molecules have been proposed to serve as upstream signals for activation of VSOR Cl^- channels, although the detailed signal pathways have not been defined. Shimizu et al. [14] demonstrated that ROS is responsible for activation of VSOR Cl^- channels during STS-induced apoptosis in HeLa cells. Their data showed that the levels of ROS significantly increased 0.5 min after application of STS, and both of ROS scavengers, *N*-acetyl-cysteine (NAC), and inhibitors of NAD(P)H oxidases, diphenylene-iodonium chloride (DPI), inhibited STS-induced VSOR Cl^- currents. A study performed by Varela et al. [28] further supported the notion. Moreover, their electrophysiological data suggested that H_2O_2, but not other ROS, is required for activation of VSOR Cl^- currents, because H_2O_2-induced VSOR Cl^- currents were abolished by peroxide-scavenging enzyme catalase, not by membrane-permeable superoxide scavenger Triton. It has to be noted that neither NAC nor DPI abolished activation of VSOR Cl^- currents induced by Fas ligand or TNF [14], implying that ROS do not mediate activation of VSOR Cl^- currents by death-receptor mediated apoptosis inducer. Alternatively, Scr-like tyrosine kinase has been reported to mediate the activation of VSOR Cl^- currents in lymphocytes stimulated with Fas ligand, because it is suppressed by tyrosine kinase inhibitor herbimycin A or by genetic deficiency of p56Lck [15].

Apoptosis is a morphologically and biologically defined form of cell death which is eventually attained as a consequence of programmed chain reactions, including cell shrinkage, activation of caspase, DNA fragmentation, thereafter formation of apoptotic bodies. Interestingly, these apoptotic events do not necessarily occurs in all cell types undergoing apoptosis. However, a universal characteristic of apoptosis is a loss of cell volume or cell shrinkage, termed AVD. In fact, the original term used to describe the distinct mode of physiological death was ''shrinking necrosis'' highlighting the unique property of cell volume loss in these dying cells [33]. However, the loss of cell volume during apoptosis has been viewed as a passive process occurring to facilitate the breakdown of the cell into smaller, apoptotic bodies. In contrast with the earlier understanding, accumulating evidence has now suggested that AVD plays an important role in regulation of programmed cell death. First, AVD is an early and active event that precedes known biochemical events. Several studies showed that AVD preceded both activation of caspase-3 and DNA fragmentation [21,34]. Particularly, Maeno et al. observed that a broad-spectrum caspase inhibitor, zVAD-fmk or zD-dcb, abolished activation of caspase-3, but not AVD in U937 cells. These results indicated that AVD is an upstream event to known biochemical apoptotic events. Second, uncontrolled changes in cell volume lead to activation of AVD event resulting in apoptosis. Bortner and Cidlowski [18] reported that in some cell types that lack effective volume mechanisms, hypertonic conditions led to sustained cell shrinkage resulting in activation of apoptosis, whereas in other cell types that maintained normal cell volume in response to hypertonic conditions, apoptosis did not occur. Maeno et al. also provided evidence that facilitated or exaggerated RVD activated AVD processes, resulting in apoptotic cell death in four different cell lines. Finally, similar to our results, prevention of AVD with Cl^- channel blockers leading to rescuing cell from apoptosis is found in some cell types [13,16,35].

Like RVD, AVD requires the efflux of intracellular solutes (K^+, Cl^-, etc.), which lead to osmotic water efflux. To produce net KCl efflux, both K^+ and Cl^- conductances must simultaneously contribute to maintaining electroneutrality. Since VSOR Cl^- channels are professional anion channels for volume regulation [36], it is not surprising that VSOR Cl^- channels are involved in AVD processes and thereby have a role in regulation of cell apoptosis. However, it is not completely known which is the most important event triggering apoptotic reactions: shrinkage itself, a $[K^+]_i$ decrease or a $[Cl^-]_i$ decrease. Intracellular acidification has been reported to occur within 3 h upon death receptor triggering in lymphocytes and precedes DNA fragmentation [37]. Moreover, Szabo et al. reported that intracellular acidification was abolished by inhibition of VSOR Cl^- channels, suggesting the involvement of VSOR Cl^- channels in CD95-induced lymphocyte apoptosis through the regulation of intracellular acidification. In contrast, clamping the cytosolic pH to an alkaline did not prevent STS-induced apoptosis in ECV304 cells [30]. A growing body of evidence has suggested that the reduction in $[K^+]_i$ provides the necessary conditions for activation of caspase and endonucleases [20,21,38]. Moreover, the dependence of K^+ channel opening on extracellular Cl^- was observed in some cell types [39]. Therefore, there is a possibility that Cl^- movement affects K^+ channel kinetics, thereby regulating $[K^+]_i$ and cell apoptosis. Our under-

standing of the mechanisms underlying regulation of apoptosis by VSOR Cl^- channels is still far from being complete and more investigations in detail are necessary for revealing the signaling pathways linking VSOR Cl^- channels to apoptosis.

There are several inherent limitations in our study. First, our study was carried out in a cultured mesangial cell line. Although functional role of ion channels has been mostly studied in a culture environment, several studies reported that functional expression of ion channels in mesangial cells may be affected by changes in phenotype or culture environment [27]. Hence, these results from such a vitro model may not be generalized to the physiological system. Second, our present study demonstrated that exogenous H_2O_2 activated VSOR Cl^- channels, but we did not provide direct evidence that endogenous H_2O_2 does so. Further studies on this issue are warranted. Third, in this study, we found that H_2O_2 activated Cl^- currents with phenotypical properties of VSOR Cl^- channels. However, the roles of other Cl^- channels in regulation of apoptotic cell death were not completely ruled out, because highly selective blockers of VSOR Cl^- channels are not available and their clone has not been identified. Finally, we suggested that the involvement of VSOR Cl^- channels in H_2O_2-induced apoptosis is associated with AVD, but our data do not reveal the precise molecular mechanisms. Hence, further studies are necessary for explaining how AVD lead to activation of apoptosis.

In summary, we demonstrated here that VSOR Cl^- channels are involved in H_2O_2-induced apoptosis in cultured mesangial cells and its mechanism is associated with AVD processes. Blocker of VSOR Cl^- channels can reduce apoptotic cell death induced by H_2O_2 in mesangial cells.

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