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TRPC6 channel activation promotes neonatal glomerular mesangial cell apoptosis via calcineurin/NFAT and FasL/Fas signaling pathways

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Glomerular mesangial cell (GMC) proliferation and death are involved in the pathogenesis of glomerular disorders. The mechanisms that control GMC survival are poorly understood, but may include signal transduction pathways that are modulated by changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration. In this study, we investigated whether activation of the canonical transient receptor potential (TRPC) 6 channels and successive $[Ca^{2+}]_i$ elevation alter neonatal GMC survival. Hyperforin (HF)-induced TRPC6 channel activation increased $[Ca^{2+}]_i$ concentration, inhibited proliferation, and triggered apoptotic cell death in primary neonatal pig GMCs. HF-induced neonatal GMC apoptosis was not associated with oxidative stress. However, HF-induced TRPC6 channel activation stimulated nuclear translocation of the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1). HF also increased cell death surface receptor Fas ligand (FasL) level and caspase-8 activity in the cells; effects mitigated by $[Ca^{2+}]_i$ chelator BAPTA, calcineurin/NFAT inhibitor VIVIT, and TRPC6 channel knockdown. Accordingly, HF-induced neonatal GMC apoptosis was attenuated by BAPTA, VIVIT, Fas blocking antibody, and a caspase-3/7 inhibitor. These findings suggest that TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation and the ensuing induction of the calcineurin/NFAT, FasL/Fas, and caspase signaling cascades promote neonatal pig GMC apoptosis.

Glomerular mesangial cell (GMC) proliferation and death are involved in the maintenance of glomerular integrity and pathophysiological mechanisms that underlie kidney dysfunctions[1]. GMC apoptosis contributes to the resolution of glomerular hypercellularity, a common characteristic of proliferative glomerulonephritis[2]. Extracellular matrix accumulation, GMC apoptosis, and glomerular sclerosis are associated with proteinuria and hypertension in diabetic nephropathy[1,3,4]. Mesangial integrity is also altered in childhood nephrotic syndrome[5]. The mechanisms that regulate GMC survival are unresolved, but may include signal transduction pathways that are modulated by changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration[6].

Ion channels, including voltage-dependent Ca^{2+}, Ca^{2+}-activated K^+, Ca^{2+}-activated Cl^-, and transient receptor potential channels are functionally expressed in GMCs[7,8]. These channels control $[Ca^{2+}]_i$ concentration and hence, Ca^{2+}-sensitive cellular events, including contraction, proliferation, and apoptosis[7,8]. The canonical transient receptor potential (TRPC) 6 has been implicated in glomerular pathophysiology[9,10]. TRPC6 channel activation alters podocyte survival and actin cytoskeleton dynamics[9,10]. Focal segmental glomerulosclerosis, an important cause of nephrotic syndrome is associated with TRPC6 channel gain of function mutations and succeeding elevation in TRPC6-dependent Ca^{2+} influx in podocytes[11,12]. An increase in Ca^{2+} influx elicited by angiotensin II-induced TRPC6 channel activation in podocytes has also been reported in diabetic nephropathy[13]. By contrast, TRPC6 channel expression and angiotensin II-induced $[Ca^{2+}]_i$ elevation are downregulated in high glucose-challenged GMCs[14]. Studies have also shown that TRPC6-mediated $[Ca^{2+}]_i$ elevation regulates angiotensin II- and phenylephrine-induced proliferation and chronic hypoxia-induced actin assembly and reorganization in GMCs[15–17]. However, the downstream targets that link TRPC6-dependent Ca^{2+} signaling to cellular events in GMCs are poorly understood.

The nuclear factor of activated T cells (NFAT) family of transcription factors includes four members whose activations are regulated by calcineurin, a Ca^{2+}-dependent protein phosphatase[18–20]. NFATs control transcription

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of a variety of genes, including those involved in cell differentiation, growth, and death[18–20]. In cardiac cells and podocytes, NFATs are targets of TRPC6-dependent $[Ca^{2+}]_i$ elevation[21–24]. However, whether NFATs are downstream effectors of TRPC6 channel activation in GMCs is unclear. Given that NFAT-regulated genes control cell survival[18–20], we examined whether a direct activation of TRPC6 channels alters neonatal GMC survival via NFAT signaling pathway. Our data suggest that hyperforin (HF)-induced TRPC6 activation inhibits proliferation and promotes apoptosis of primary neonatal pig GMCs. We also show that TRPC6-mediated neonatal GMC apoptosis is associated with an induction of the cell death surface receptor Fas ligand (FasL) and caspase-8 by NFATc1. Collectively, we provide a novel insight into the mechanisms by which TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation and sequential activation of the calcineurin/NFAT and FasL/Fas signaling pathways stimulate neonatal pig GMC apoptosis.

Results

HF-induced TRPC6 channel activation elevates $[Ca^{2+}]_i$ in neonatal GMCs. TRPC6 channels regulate $[Ca^{2+}]_i$ concentration in rat and human GMCs[14–17]. To confirm that activation of TRPC6 channels stimulates Ca^{2+} influx in neonatal pig GMCs, we studied the effect of HF, a TRPC6 channel activator[25–30] on $[Ca^{2+}]_i$ concentration in the cells. First, we examined whether HF stimulates Ca^{2+} release from intracellular Ca^{2+} stores in the cells. In the absence of extracellular Ca^{2+}, HF did not alter basal $[Ca^{2+}]_i$ in the cells (Fig. 1a). However, successive re-addition of extracellular Ca^{2+} in the continued presence of HF resulted in an increase in $[Ca^{2+}]_i$ by 186.7 ± 3.4 nM (n = 3; Fig. 1a). By contrast, in the absence of extracellular Ca^{2+}, protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) elevated $[Ca^{2+}]_i$ by 64.4 ± 9.2 nM (n = 5; Fig. 1a) in the cells. Restoration of extracellular Ca^{2+} in the presence of CCCP did not stimulate a further increase in $[Ca^{2+}]_i$ (29.2 ± 6.0 nM; n = 5; $P < 0.05$ vs. absence of extracellular Ca^{2+}; Fig. 1a). Next, we knocked down TRPC6 channels and examined the effect of HF on $[Ca^{2+}]_i$ concentration in the cells. As shown in Fig. 1b,c, TRPC6 siRNA reduced TRPC6 protein expression by ~66%. HF increased $[Ca^{2+}]_i$ by ~197 nM in control cells (Fig. 1d,e). However, TRPC6 channel knockdown significantly reduced HF-induced $[Ca^{2+}]_i$ elevation (Fig. 1d,e). These data indicate that HF does not stimulate Ca^{2+} release from intracellular organelles, but elevates $[Ca^{2+}]_i$ by activating membrane-resident TRPC6 channels in neonatal pig GMCs.

TRPC6 channel activation inhibits proliferation and promotes apoptosis of neonatal GMC. Changes in $[Ca^{2+}]_i$ regulate several intracellular signaling pathways that promote GMC proliferation and death[6,31]. Data here indicate that HF stimulates $[Ca^{2+}]_i$ elevation via TRPC6 channels (Fig. 1). To study the effect of HF on GMC survival, we measured the time-course of proliferation and apoptotic death in the cells. The CellPlayer caspase-3/7 green fluorescence reagent is non-fluorescent in non-apoptotic cells. However, activation of caspase activity during apoptosis increases the fluorescent signal. As shown in Fig. 2a,b, HF inhibited neonatal pig GMC confluence in a time- and concentration-dependent manner. At the same time, HF increased caspase-3/7 activity in the cells (Fig. 2c,d). At 1 and 3 μM, HF inhibited GMC proliferation but did not significantly induce apoptosis (Fig. 2b,d). To confirm that TRPC6 channels mediate HF-induced neonatal pig GMC apoptosis, we measured caspase-3/7 activity in cells transfected with a non-targeting control and TRPC6 channel siRNAs. As shown in Fig. 2e,f, knockdown of TRPC6 channels attenuated HF-induced caspase-3/7 activation in the cells. Together, these data suggest that HF-induced TRPC6 channel activation inhibits proliferation and stimulates apoptosis of neonatal pig GMCs.

HF-induced neonatal GMC apoptosis is not associated with oxidative stress. To investigate the role of oxidative stress in HF-induced neonatal GMC apoptosis, we first measured reactive oxygen species (ROS) generation in control and neonatal pig GMCs treated with HF. Confocal microscopy showed that pyocyanin, a superoxide inducer stimulates superoxide generation in neonatal pig GMCs (Fig. 3a). By contrast, neither DMSO (control) nor HF caused significant superoxide generation in the cells (Fig. 3a). Thiobarbituric acid reactive substance (TBARS) assay indicated that unlike oxidant tert-butyl hydroperoxide (TBHP), HF did not increase malondialdehyde (MDA; an end product of lipid peroxidation) in the cells (Fig. 3b). Whereas HF did not alter H_2O_2 level, TBHP elevated H_2O_2 production in the cells (Fig. 3c). Next, we examined whether inhibition of oxidative stress mitigates HF-induced neonatal pig GMC apoptosis. As shown in Fig. 3d,e, TBHP promoted rapid apoptosis of the cells. TEMPOL, a cell permeable ROS scavenger essentially abolished TBHP-induced GMC apoptosis (Fig. 3d,e). By contrast, TEMPOL did not alter HF-induced apoptosis of the cells (Fig. 3f,g). These findings indicate that HF-induced neonatal pig GMC apoptosis is not associated with oxidative stress.

TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation stimulates nuclear translocation of NFATc1 in neonatal GMCs. To examine whether HF-induced $[Ca^{2+}]_i$ elevation via TRPC6 channels stimulates nuclear translocation of NFATc1 in neonatal GMCs, we studied the localization of a GFP-NFATc1 cDNA clone that was transiently transfected into neonatal pig GMCs. Unlike DMSO (control)-treated cells, GFP-NFATc1 showed predominant nuclear localization in HF-treated cells (Fig. 4a,b). GFP-NFATc1 nuclear localization in the cells was significantly reduced by BAPTA, an $[Ca^{2+}]_i$ chelator and VIVIT, a calcineurin/NFAT inhibitor (Fig. 4a,b). Also, GFP-NFATc1 nuclear localization was attenuated by TRPC6 channel knockdown (Fig. 4a,c). These data suggest that HF-induced $[Ca^{2+}]_i$ elevation via TRPC6 channels elicits nuclear translocation of NFATc1 in neonatal pig GMCs.

TRPC6 channel-mediated $[Ca^{2+}]_i$ elevation increases FasL level and caspase-8 activity in neonatal GMCs. FasL/Fas interaction at the cell surface is a major contributor to death-inducing signaling pathways in many cell types, including GMCs[32,33]. FasL/Fas interaction triggers caspase-8 and -3 activation[32]. Our data indicate that HF-induced TRPC6 activation stimulates NFATc1 nuclear localization in neonatal pig

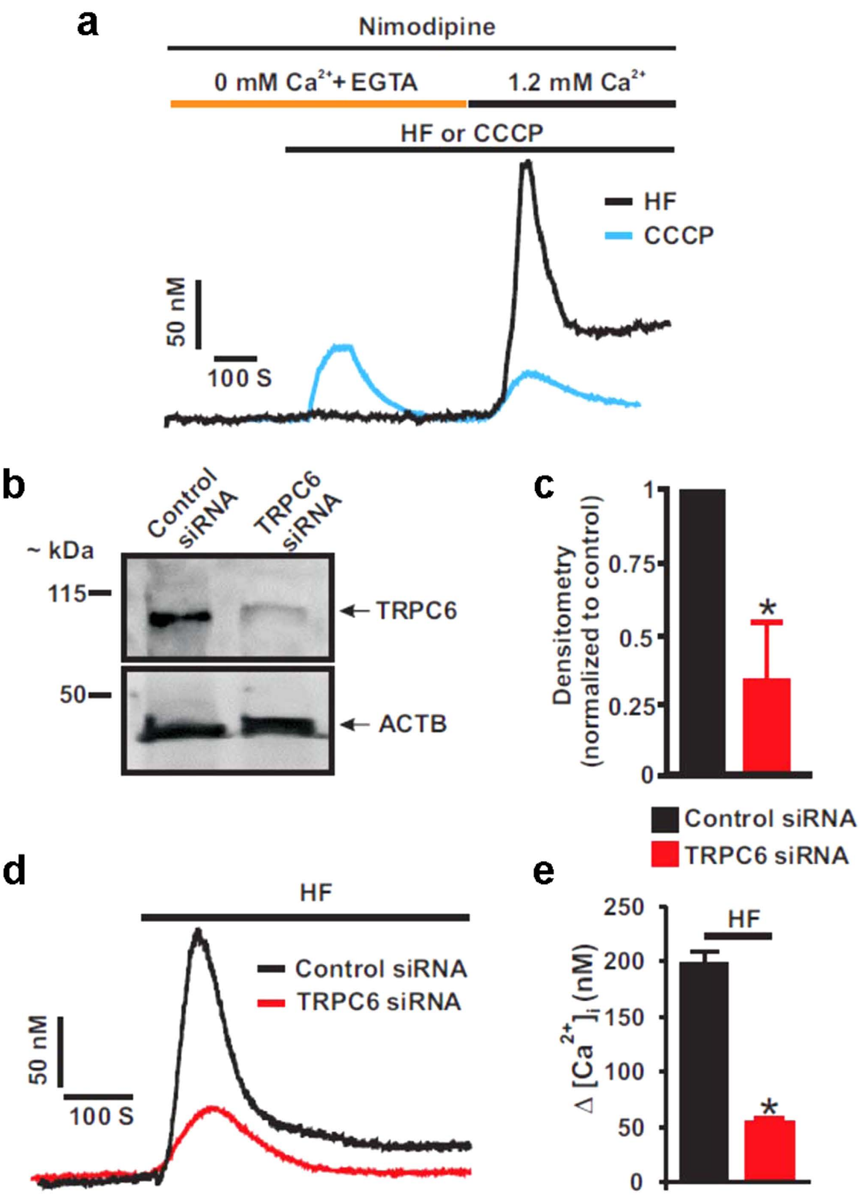

Figure 1. HF-induced TRPC6 channel activation elevates $[Ca^{2+}]_i$ in neonatal GMCs. (**a**) Exemplar traces showing the effects of HF and CCCP on $[Ca^{2+}]_i$ concentration in the absence and presence of extracellular Ca^{2+} in neonatal pig GMCs. The bathing solution was supplemented with EGTA (0.1 mM) and nimodipine (1 μM) to chelate Ca^{2+} and block L-type Ca^{2+} channels, respectively. (**b,c**) Western blot image and mean data (n = 3 each) confirming siRNA-mediated knockdown of TRPC6 channels. (**d,e**) Traces and mean data indicating that TRPC6 channel knockdown attenuates HF (10 μM)-induced $[Ca^{2+}]_i$ elevation in neonatal GMCs (n = 4 each). *P < 0.05 vs. control siRNA.

GMCs (Fig. 4). Given that NFATs are involved in transcriptional regulation of FasL expression[34,35], we reasoned that TRPC6-depedent NFATc1 activation may elevate membrane FasL level and stimulate caspase-8 activity in neonatal GMCs. Hence, we used a porcine FasL ELISA kit to quantify the level of FasL in neonatal pig GMC lysates. We also measured caspase-8 activity in the cell lysates. As shown in Fig. 5a, FasL level was increased in neonatal pig GMCs treated with HF. HF-induced increase in FasL was diminished in cells pretreated with BAPTA and VIVIT (Fig. 5a). siRNA-mediated knockdown of TRPC6 channels also reduced HF-induced increase in FasL level (Fig. 5b). Similarly, HF treatment elevated caspase-8 activity in neonatal pig GMCs (Fig. 5c). HF-induced elevation in caspase-8 activity was significantly attenuated by BAPTA, VIVIT, and TRPC6 channel knockdown (Fig. 5c,d). To examine the role of FasL/Fas activation in HF-induced caspase-8 activity, we blocked FasL/Fas interaction with a Fas blocking antibody (Fas BA). As shown in Fig. 5c, there was no significant change in caspase-8 activity in GMCs, pretreated with Fas BA before HF application. These findings suggest that TRPC6-dependent $[Ca^{2+}]_i$ elevation in neonatal pig GMCs increases FasL and caspase-8 activity

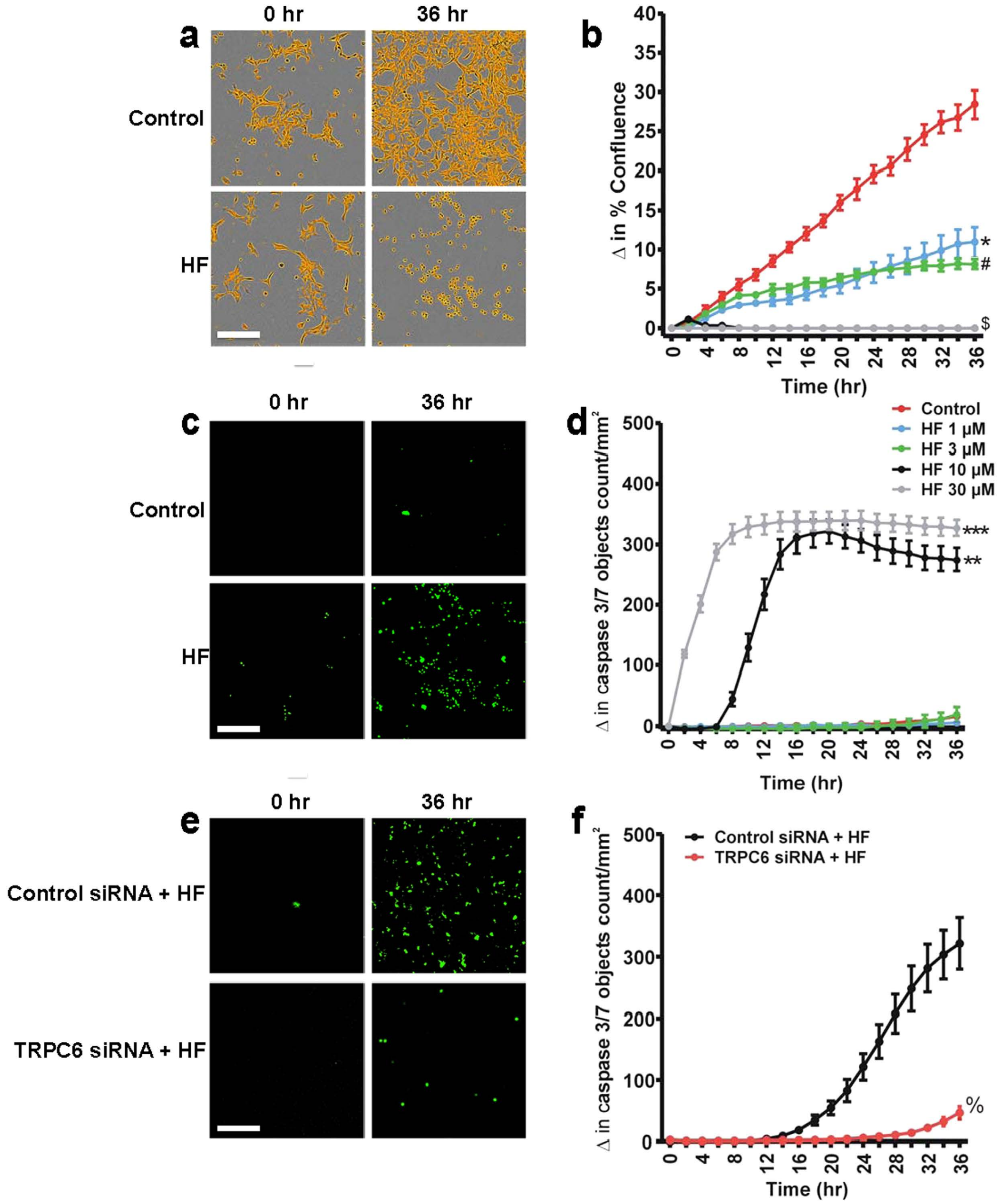

Figure 2. TRPC6 channel activation inhibits proliferation and promotes apoptosis of neonatal GMCs. (**a**) Phase contrast images (segmentation mask illustrated in goldenrod; HF, 10 µM) and (**b**) Cell growth curves showing time- and concentration-dependent anti-proliferative effect of HF (n = 4 each) in neonatal pig GMCs. (**c**) Images (green fluorescent staining of nuclear DNA in apoptotic cells; HF, 10 µM) and (**d**) Kinetic curves demonstrating that HF (n = 4 each) induces time- and concentration-dependent increase in caspase-3/7 activity in neonatal pig GMCs. (**e**) Images and (**f**) Kinetic curves illustrating that TRPC6 channel knockdown attenuates HF (10 µM; n = 4 each)-induced caspase-3/7 activity in neonatal pig GMCs. $^{\#}$P < 0.05 vs. control (DMSO; 12–36 hr); *P < 0.05 vs. control (DMSO; 10–36 hr); $^{\$}$P < 0.05 vs. control (DMSO; 6–36 hr); **P < 0.05 vs. control (DMSO; 10–36 hr); ***P < 0.05 vs. control (DMSO; 2–36 hr); $^{\%}$P < 0.05 vs. control siRNA (20–36 hr); Scale bars = 100 µm.

via calcineurin/NFAT signaling. Our data also demonstrate that FasL/Fas interaction regulates HF-induced caspase-8 activation in the cells.

TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation and successive activation of the NFAT and FasL/Fas signaling pathways trigger neonatal GMC apoptosis. To confirm that TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation and successive activation of the extrinsic apoptotic signaling pathway induce neonatal GMC apoptosis, we measured HF-induced caspase-3/7 activation in neonatal pig GMCs pretreated with DMSO

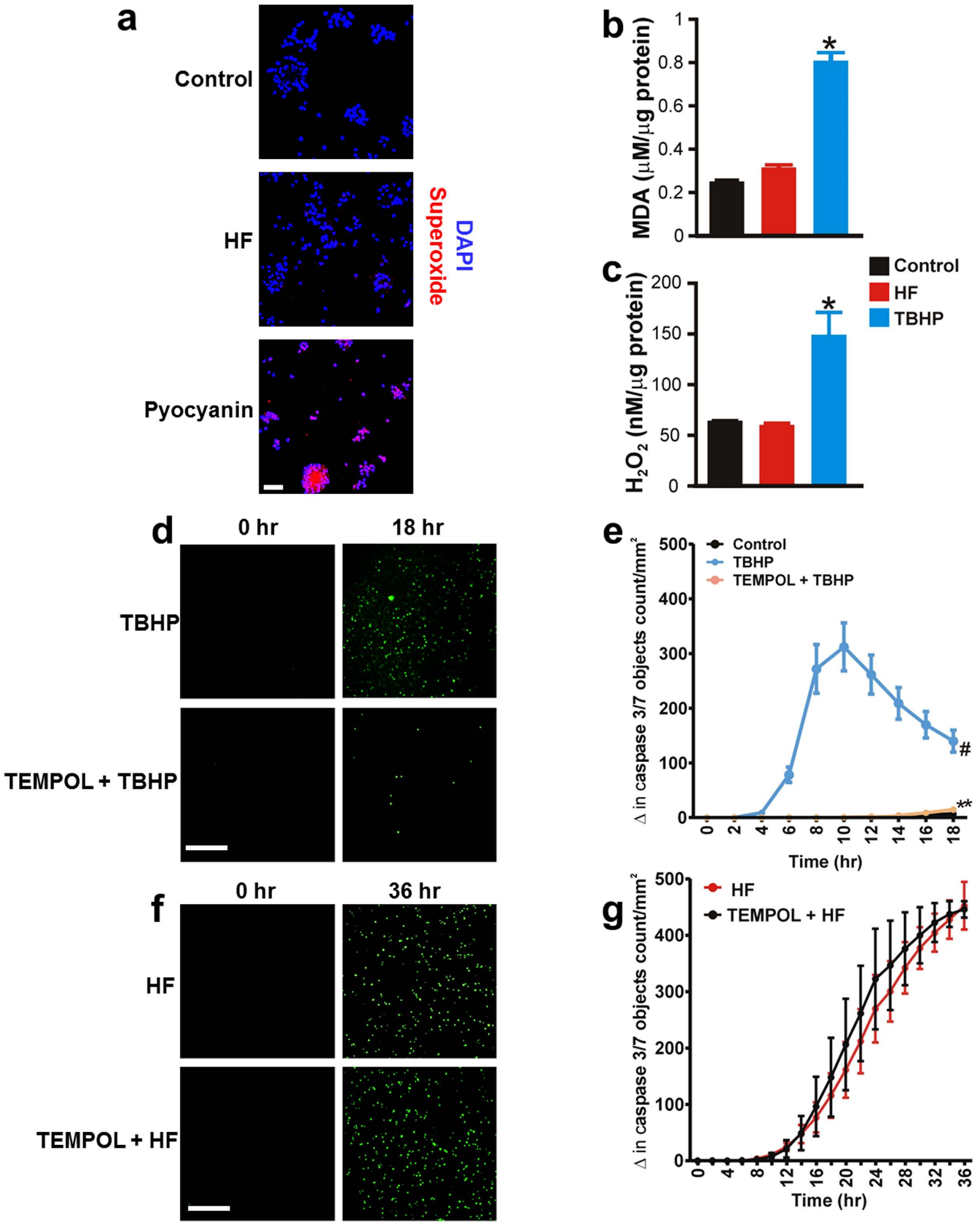

Figure 3. HF-induced neonatal GMC apoptosis is not associated with oxidative stress. (**a**) Representative confocal images showing that superoxide production was detected in GMCs treated with ROS inducer pyocyanin (10 μM; 8 hr)-, but not in HF (10 μM; 8 hr)-treated cells (images were obtained from 3 coverslips per experimental condition). (**b**) Mean data illustrating that unlike tert-butyl hydroperoxide (TBHP; 10 μM; 8 hr), HF (10 μM; 8 hr) does not increase MDA production in neonatal pig GMCs (n = 4 each). (**c**) Mean data showing cellular H_2O_2 concentration in DMSO (control)-, HF (10 μM; 8 hr)- and TBHP (10 μM; 8 hr)-treated cells (n = 5 each). (**d**) Images (green fluorescent staining of nuclear DNA in apoptotic cells) and (**e**) Kinetic curves indicating that TBHP (10 μM; n = 5 each)-induced caspase-3/7 activity in neonatal pig GMCs is inhibited by TEMPOL. (**f**) Images and (**g**) Kinetic curves demonstrating that HF (10 μM; n = 4 each)-induced caspase-3/7 activity in neonatal pig GMCs is unaltered by TEMPOL. *P < 0.05 vs. DMSO and HF; #P < 0.05 vs. Control (PBS; 6–18 hr); **P < 0.05 vs. TBHP (6–18 hr); Scale bars = 100 μm.

(control), BAPTA, and inhibitors of NFAT, Fas, and caspase-3/7. As shown in Fig. 6, HF-induced caspase-3/7 activation was mitigated by BAPTA, VIVIT, FK506 (a calcineurin inhibitor), Fas BA, and Ac-DEVD-CHO (a caspase-3/7 inhibitor). Together, these data demonstrate that an elevation in $[Ca^{2+}]_i$ elicited by HF-induced TRPC6 channel activation, and sequential stimulation of the calcineurin/NFAT, FasL/Fas, and caspase signaling pathways promote neonatal GMC apoptosis (Fig. 7).

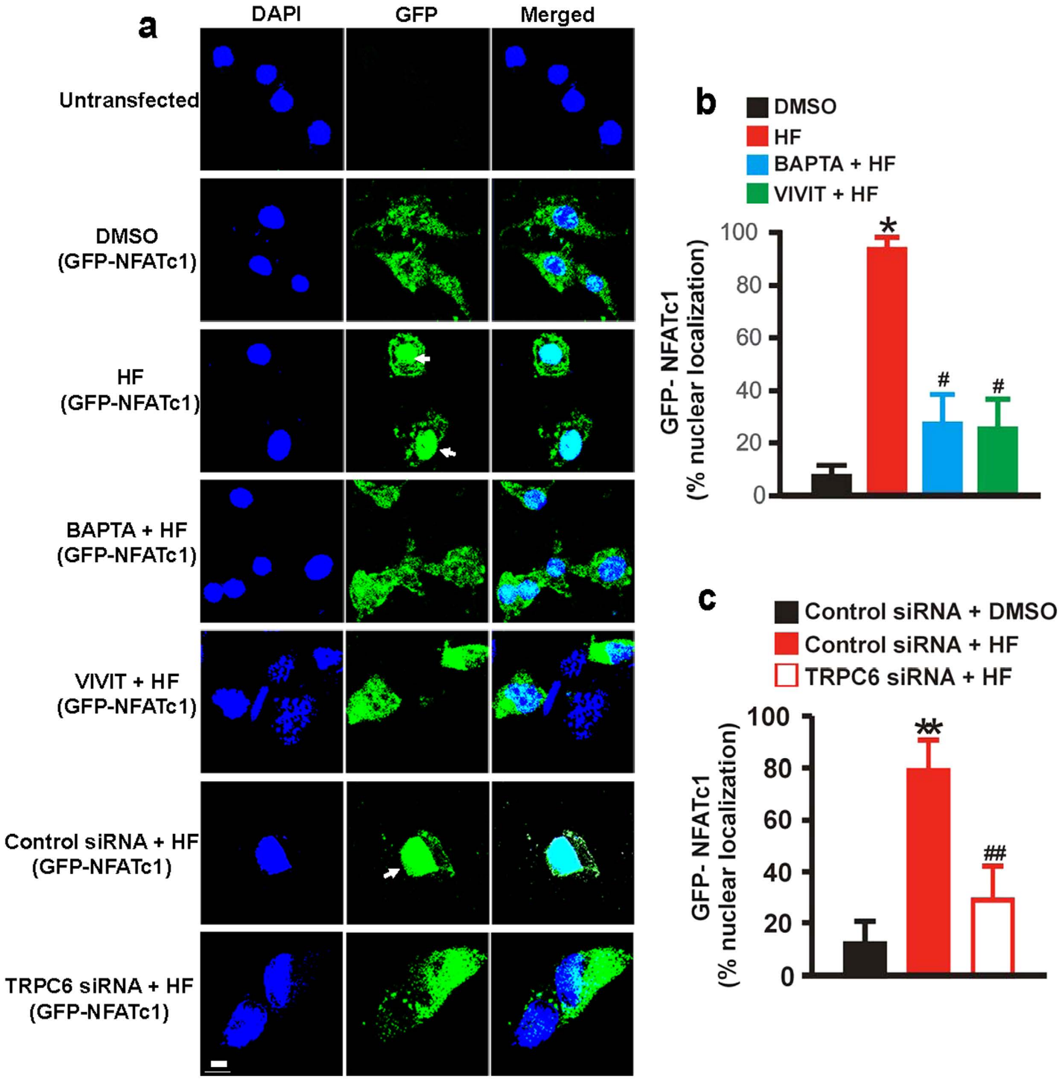

Figure 4. TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation stimulates nuclear translocation of NFATc1 in neonatal GMCs. (**a**) Confocal microscopy images, and (**b,c**) Mean data showing that HF (10 μM; 1 hr) stimulates nuclear translocation of GFP-tagged NFATc1 in neonatal pig GMCs and that BAPTA (1 μM), VIVIT (1 μM), and TRPC6 channel knockdown inhibit HF-induced NFATc1 nuclear translocation (n = 9, DMSO, HF, BAPTA + HF, and VIVIT + HF; n = 10, control siRNA + DMSO; n = 12 control siRNA + HF and TRPC6 siRNA + HF image fields from 3 coverslips per experimental condition). Cells were pretreated with blockers/inhibitors for ~30 min before the addition of HF. $^*P < 0.05$ vs. DMSO; $^\#P < 0.05$ vs. HF; $^{**}P < 0.05$ vs. control siRNA + DMSO; $^{\#\#}P < 0.05$ vs. control siRNA + HF; Sacle bar = 20 μm. White arrows indicate NFATc1 nuclear localization.

Discussion

The fine-tuning of the expression and function of ion channels and second messengers that maintain $[Ca^{2+}]_i$ concentration underlies a variety of signaling events that control cell survival[6]. HF, a phloroglucinol derivative and selective TRPC6 channel activator elevated $[Ca^{2+}]_i$ in neuronal, vascular smooth muscle, and dermal cells and cardiac fibroblast[25–28]. HF impedes tumor cell growth by inducing caspase-dependent apoptosis[36,37]. However, modulation of GMC survival by HF-induced TRPC6 activation has not been previously investigated. In this study, we show for the first time that activation of TRPC6 channels by HF increases $[Ca^{2+}]_i$ concentration and induces NFAT-, FasL/Fas-, and caspase-dependent neonatal pig GMC apoptosis.

The control of mesangial $[Ca^{2+}]_i$ homeostasis involves TRPC channel-mediated signal transduction pathways[8,38]. TRPC channels, comprising of seven members (TRPC1-7), can assemble as homo- or hetero-tetramers to form non-selective cation channels that are activated by multiple signaling modalities, including membrane stretch, G-protein-coupled receptor stimulation, and $[Ca^{2+}]_i$ store depletion[39,40]. Previous studies have shown that TRPC6 channels participate in GMC proliferation associated with angiotensin II- and phenylephrine-induced store- and receptor-operated Ca^{2+} entry in rat and human GMC lines[15,16]. Here, simultaneous measurement of cell confluence metrics and caspase-3/7 activity in real-time indicated that a direct activation of TRPC6 channels inhibits proliferation and triggers apoptosis of primary neonatal pig GMCs. The conflicting data on the effect of

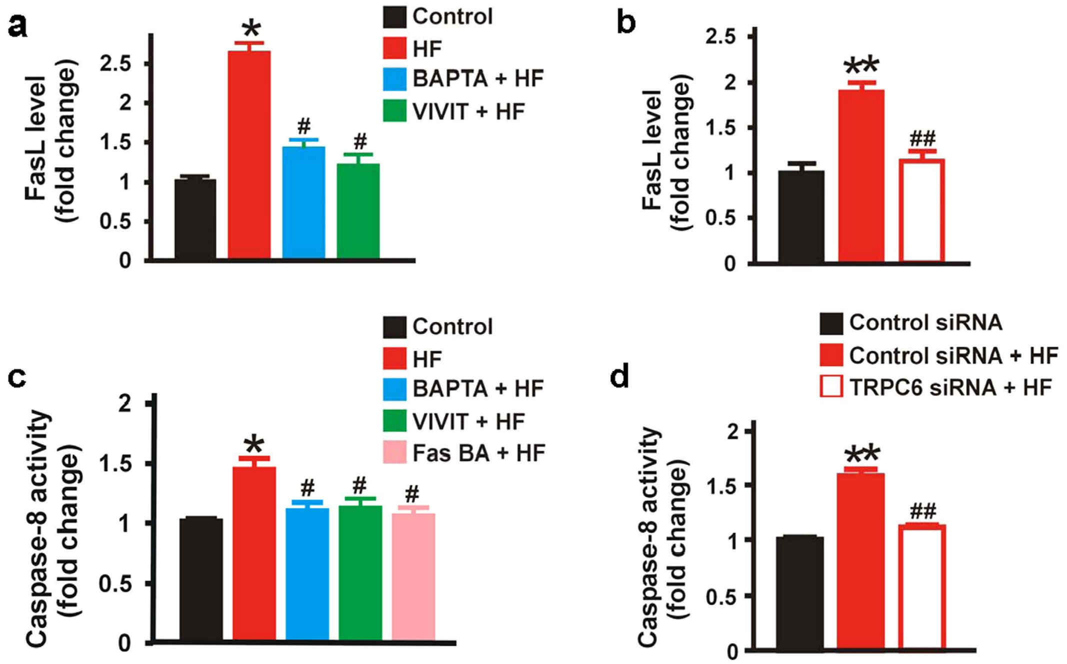

Figure 5. TRPC6 channel-mediated $[Ca^{2+}]_i$ elevation increases FasL level and caspase-8 activity in neonatal GMCs. (**a**) Mean data obtained from porcine FasL ELISA assay demonstrating that HF (10 μM; 8 hr) elevates FasL level in neonatal pig GMCs and that HF-induced FasL is abrogated by BAPTA (1 μM) and VIVIT (1 μM); n = 6 each. (**b**) Mean data showing that HF (10 μM; 8 hr)-induced FasL elevation in neonatal pig GMCs is reduced by siRNA-mediated TRPC6 channel knockdown; n = 6 each. (**c**) Mean data obtained from caspase-8 colorimetric assay indicating that HF (10 μM; 8 hr) increases caspase-8 activity in neonatal GMCs, and that BAPTA (1 μM), VIVIT (1 μM), and Fas blocking antibody (Fas BA; 10 μg/mL; n = 4 each) essentially abolish HF-induced increase in caspase-8 activity. (**d**) Mean data showing that HF (10 μM; 8 hr)-induced caspase-8 activity in neonatal pig GMCs is attenuated by siRNA-mediated TRPC6 channel knockdown (n = 4, control siRNA and control siRNA + HF; n = 5, TRPC6 siRNA + HF). Cells were pretreated with blockers/inhibitors for ~30 min before the addition of HF. Data are expressed as fold change relative to the control. $^*P < 0.05$ vs. DMSO; $^{\#}P < 0.05$ vs. HF; $^{**}P < 0.05$ vs. control siRNA; $^{\#\#}P < 0.05$ vs. control siRNA + HF.

TRPC6 activation on GMC growth may be related to kidney maturation or the different mechanisms of channel activation. Whether $[Ca^{2+}]_i$ elevation induces GMC proliferation or death may also depend on downstream cellular signaling pathways that are activated[6].

To investigate the mechanisms that mediate HF-induced neonatal GMC apoptosis, we tested the hypothesis that HF promotes oxidative stress in the cells. Our data show that at the concentration that elicited apoptotic cell death, HF did not stimulate lipid peroxidation and superoxide and H_2O_2 production in the cells. Furthermore, at the concentration that abrogated TBHP-induced apoptosis, ROS scavenger TEMPOL did not alter HF-induced apoptosis of the cells. Hence, oxidative damage is an unlikely mechanism of HF-induced neonatal pig GMC apoptosis.

The NFAT family of transcription factors are important regulators of genes that promote differentiation, proliferation and death in a broad range of cells[18–20]. In resting cells, phosphorylated NFATs are mostly localized in the cytoplasm, and are activated by calcineurin, a Ca^{2+}/calmodulin (CaM)-dependent serine/threonine phosphatase[18–20]. Following an elevation in $[Ca^{2+}]_i$, the Ca^{2+}-CaM complex activates calcineurin. NFAT proteins are then directly dephosphorylated by activated calcineurin, resulting in their nuclear translocation and induction of NFAT-mediated gene transcription[18–20]. In cardiac cells, NFAT stimulation and the resultant pathological hypertrophy are associated with $[Ca^{2+}]_i$ increase caused by the activation of TRPC1, TRPC3, TRPC4, and TRPC6 channels[22,41,42]. In the kidney, TRPC6 channel-mediated activation of NFATc1-dependent gene transcription is involved in glomerulosclerosis[23,24]. Tumor necrosis factor- and angiotensin II-induced activation of NFATc1 can also increase TRPC6 channel expression in podocytes[21,43]. Here, we show for the first time that activation of TRPC6 channels in neonatal GMCs stimulates NFATc1 nuclear translocation in a Ca^{2+}-dependent fashion. HF-induced apoptotic cell death was also reduced in neonatal GMCs pretreated with calcineurin/NFAT inhibitors FK506 and VIVIT. These findings indicate that NFAT is a downstream target of TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation in GMCs. The NFAT family consists of four Ca^{2+}/calcineurin-responsive members NFATc1, NFATc2, NFATc3, and NFATc4[18–20]. Thus, activation of multiple NFAT members by TRPC6-mediated $[Ca^{2+}]_i$ elevation in GMCs is possible, but not demonstrated in this study.

Activation of the cell death surface receptor, Fas by its cognate ligand FasL is a major contributor to the extrinsic pathway of programmed cell death[32,34,44]. FasL/Fas interaction recruits the adapter protein Fas-associated protein with death domain, which in turn employs its death effector domain to induce procaspase-8 and -10[45–47].

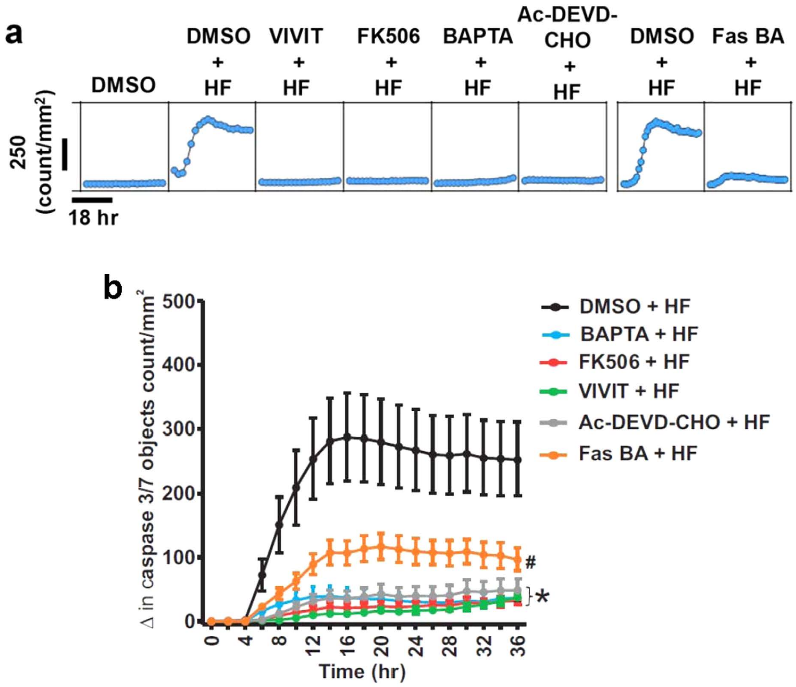

Figure 6. TRPC6 channel-dependent $[Ca^{2+}]_i$ elevation and successive activation of the NFAT and FasL/Fas signaling pathways trigger neonatal GMC apoptosis. (**a**) Representative microplate graphs and (**b**) Kinetic curves showing HF-induced caspase-3/7 activity in control and BAPTA (1 μM; n = 10)-, FK506 (100 nM; n = 10)-, VIVIT (1 μM; n = 10)-, Ac-DEVD-CHO (50 μM; n = 10)-, and Fas BA (10 μg/mL; n = 5)-treated GMCs. Cells were pretreated with blockers/inhibitors for ~30 min before the addition of HF. $^*P < 0.05$ vs. control (8–36 hr); $^{\#}P < 0.05$ vs. control (10–36 hr).

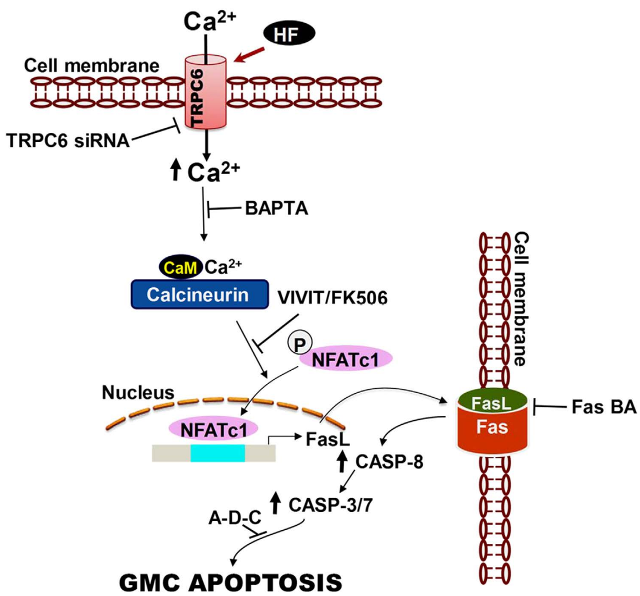

Figure 7. Schematic diagram illustrating hypothetical mechanisms by which an elevation in $[Ca^{2+}]_i$ as a result of TRPC6 channel activation, and subsequent induction of the calcineurin/NFAT, FasL/Fas, and caspase signaling cascades promote neonatal GMC apoptosis. CASP (caspase); Ac-DEVD-CHO (A-D-C); BA (blocking antibody); CaM (calmodulin).

Formation of the death-inducing signaling complex as a result of clustering and subsequent autocatalysis of procaspase-8 and -10 triggers apoptosis via downstream executioner caspases, including caspase-3 and -7[45,47,48]. The expression of FasL is controlled by transcription factors such as nuclear factor-kappa B, early growth response protein, and NFAT[34,35]. NFAT-induced FasL expression has been implicated in Jurkat T, breast cancer, cardiac, neuronal, and Leydig cell apoptosis[49–52], but whether NFAT regulates cell death induced by the FasL/Fas pathway in GMCs was unclear. Data here show that TRPC6 activation elevates membrane FasL level and caspase-8 activity in neonatal pig GMCs, and these effects were essentially abolished by $[Ca^{2+}]_i$ chelation, calcineurin/NFAT inhibition, and TRPC6 channel knockdown. These data suggest the involvement of TRPC6-dependent calcineurin/NFAT activation in FasL and caspase-8 induction in neonatal pig GMCs. HF-induced caspase-3/7 activity was also attenuated by $[Ca^{2+}]_i$ chelation, calcineurin/NFAT inhibition, and Fas blockade. Together, these findings indicate that TRPC6 channel-mediated $[Ca^{2+}]_i$ elevation and subsequent activation of calcineurin/NFAT, FasL/Fas, and caspase cascades result in neonatal pig GMC apoptosis.

Studies have suggested that HF exhibit protonophore activity, and may stimulate Ca^{2+} release from the mitochondria, independently of TRPC6 channels[53–55]. Protonophores, including CCCP, inhibit mitochondrial Ca^{2+} uptake and induce mitochondrial Ca^{2+} release, thereby increasing $[Ca^{2+}]_i$[56,57]. Here, we show that in neonatal pig GMCs, CCCP elevated $[Ca^{2+}]_i$ which was independent of extracellular Ca^{2+}, suggesting Ca^{2+} release from the intracellular stores. By contrast, HF did not stimulate Ca^{2+} release from the intracellular stores but induced nimodipine-insensitive and TRPC6-dependent Ca^{2+} influx in the cells. HF-induced NFATc1 nuclear translocation, FasL and caspase 8 activation, and apoptosis of neonatal pig GMCs were also dependent on TRPC6 channels. These data suggest that TRPC6 channels mediate the effects of HF reported in the present study. Perhaps, the molecular mechanisms underlying the pharmacological actions of HF is dependent on cell type. Also, data in this study were derived from primary neonatal GMCs. Thus, TRPC6 channel-mediated intracellular signaling may differ in adult GMCs.

Whereas the contribution of TRPC6 channels to podocyte pathology is well known, their role in mesangial pathophysiology is poorly understood. Given the involvement of GMC apoptosis in glomerulopathy, we propose that mesangial derangement promoted by TRPC6-dependent $[Ca^{2+}]_i$ elevation and subsequent cell death may be of importance in renal dysfunctions, especially in immature kidneys.

Methods

Primary GMC culture. Animal welfare and use in this study were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by and carried out in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center (UTHSC). Male newborn pigs (1.5–2 kg) were purchased from Nichols Hog Farm (Olive Branch, MS) and maintained at the UTHSC Comparative Medicine Department. Renal glomeruli were isolated from the pigs (<1-week-old) by serial sieving of renal cortical homogenates using sterile stainless steel meshes. GMCs were then cultured from decapsulated glomeruli as we have previously described[58].

Western immunoblotting. Cultured GMCs were homogenized in ice-cold RIPA buffer supplemented with a protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Following isolation, proteins were separated and detected as we have previously described[58,59].

siRNA and cDNA transfection. GMC suspensions were electroporated in single cuvettes containing pre-warmed culture medium and a pool of 2 target-specific TRPC6 siRNAs or a non-targeting control siRNA (Sigma-Aldrich, St. Louis, MO). An electrical field to induce siRNA transfection was applied using the ECM 830 square wave electroporation system (Harvard Apparatus, Holliston, MA). Ten minutes after electroporation, the cells were plated for ~72 hours. Western immunoblotting was used to confirm efficient knockdown. GFP-tagged human nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1; OriGene Technologies, Rockville, MD) cDNA clone (400 ng/mL) was transfected into GMCs using the TurboFect transfection reagent (Life Technologies, Grand Island, NY). Transfected cells were maintained in 5% CO_2 humidified incubator for 48 h at 37 °C. Images were acquired using a Zeiss laser-scanning confocal microscope.

$[Ca^{2+}]_i$ imaging. Changes in $[Ca^{2+}]_i$ concentrations were measured in GMCs loaded with fura-2-acetoxymethyl ester using a ratiometric fluorescence system (Ionoptix Corp., Milton, MA, USA) as we have previously described[58,59]. To examine whether HF and CCCP induce Ca^{2+} release from intracellular stores, cells were incubated in Ca^{2+} free modified Krebs' solution[58,59] supplemented with 0.1 mM EGTA (a calcium chelator) and L-type Ca^{2+} channel blocker nimodipine (1 μM).

Live content microscopy of GMCs. Real-time growth and kinetic quantification of apoptosis in GMCs seeded in microplates (5×10^3 cells/well) were performed using CellPlayer caspase-3/7 reagent (Essen BioScience, Ann Arbor, MI) and the IncuCyte ZOOM live content microscopy system (Essen BioScience). Cell growth and kinetic activation of caspase-3/7 were monitored in real-time and automatically acquired at two-hourly intervals by the IncuCyte interface and software.

Reactive oxygen species (ROS) determination. Lipid peroxidation was evaluated in cell lysates using the TBARS kit (Thermo Scientific, Rockford, IL, Cat. No. KA1381). Superoxide generation was examined using a superoxide fluorogenic kit that specifically detects superoxide levels in live cells (Enzo Life Sciences, Farmingdale, NY; Cat. No. ENZ-51012). The fluorescence generated by this probe was visualized and documented using a Zeiss laser-scanning confocal microscope. H_2O_2 concentration in the cells was measured using a colorimetric detection kit (Arbor Assay, Ann Arbor, MI, Cat. No. K034-H1).

FasL and caspase-8 assays. GMCs were cultured in 6-well microplates as described above. Membrane FasL concentration and caspase-8 activity were quantified in cell lysates using the porcine FasL ELISA (NeoScientific, Woburn, MA, Cat. No. PF0058) and FLICE/caspase-8 colorimetric assay (BioVision, Inc. Milpitas, CA, Cat. No. K113-25) kits. Data obtained from the cell lysates were normalized to protein concentrations.

Antibodies and chemicals. Mouse monoclonal anti-actin (Cat. No. ab3280) and rabbit polyclonal anti-TRPC6 (Cat. No. ab81111) primary antibodies and HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Abcam (Cambridge, MA). DMSO, hyperforin, and tert-Butyl hydroperoxide were purchased from Sigma-Aldrich. BAPTA, FK506, VIVIT, Ac-DEVD-CHO, Fas blocking antibody, ionomycin, pluronic F-127, and Fura-2 AM were purchased from Assay Biotechnology (Sunnyvale, CA), Cell Signaling Technology (Danvers, MA), Cayman Chemical Company (Ann Arbor, MI), Biotium Inc. (Hayward, CA), ProSpec-Tany TechnoGene Ltd (Rehovot, Israel), Cayman Chemical Company, Anaspec (Fremont, CA), and Life Technologies, respectively.

Data analysis. All data are expressed as mean ± standard error of mean (SEM). Statistical significance was determined using Student's t-tests for paired or unpaired data and analysis of variance with Bonferroni post hoc test for multiple comparisons. A P value < 0.05 was considered significant.

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Author Contributions

H.S. and A.A. designed and conducted the experiments and analyzed the data. A.A. conceived the study, directed the project, and wrote the manuscript.

Additional Information

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