Signaling pathways from membrane lipid rafts to JNK1 activation in reactive nitrogen species-induced non-apoptotic cell death

Y-T Wu[1], S Zhang[1], Y-S Kim[2], H-L Tan[1], M Whiteman[3], C-N Ong[1], Z-G Liu[2], H Ichijo[4] and H-M Shen*,[1]

At present, the signaling pathways controlling reactive nitrogen species (RNS)-induced non-apoptotic cell death are relatively less understood. In this work, various RNS donors are found to induce caspase-independent non-apoptotic cell death in mouse embryonic fibroblasts (MEF). In search of the molecular mechanisms, we first established the role of c-Jun N-terminal kinase (JNK) in RNS-induced non-apoptotic cell death. RNS readily activate JNK, and the *jnk1*−/− MEF are resistant to RNS-induced cell death. Moreover, the reconstitution of JNK1 effectively restores the sensitivity to RNS. Next, we identified tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) as the essential upstream molecules for RNS-induced JNK activation and cell death. RNS fail to activate JNK and induce cell death in *traf2*−/− MEF; and reconstitution of TRAF2 effectively restores the responsiveness of *traf2*−/− MEF to RNS. Moreover, RNS-induced ASK1 activation is impaired in *traf2*−/− cells and overexpression of a mutant ASK1 protein suppresses RNS-induced cell death in wild-type MEF cells. Last, we explored the signaling events upstream of TRAF2 and found that translocation of TRAF2 and JNK1 onto membrane lipid rafts is required for RNS-mediated JNK1 activation and cell death. Taken together, data from our study reveal a novel signaling pathway regulating RNS-induced JNK1 activation and non-apoptotic cell death.
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Reactive nitrogen species (RNS) refer to various nitrogenous products including nitric oxide (NO), peroxynitrite (ONOO⁻) and nitrogen dioxide (NO_2), which are highly reactive and capable of inducing nitrosative stress and impairing the function of their target molecules, including protein, lipid and DNA.[1] Among the wide range of bioactivities, one important aspect of RNS is their regulatory roles in cell death. Under different circumstances, RNS are capable of inducing either apoptosis or non-apoptotic cell death in a variety of cells.[2] It has been well studied that RNS-induced apoptosis is executed via the following two main mechanisms: (i) mitochondria-dependent intrinsic apoptosis pathway and (ii) DNA damage and p53 activation.[3] In contrast, the signalling pathways controlling RNS-induced non-apoptotic or necrotic cell death are less studied. There is evidence suggesting that RNS impair the respiratory function of mitochondria and ATP production and eventually lead to necrosis.[4]

c-Jun N-terminal kinase (JNK) (also known as stress-activated protein kinase) is an important subgroup of the mitogen-activated protein kinases (MAPK) superfamily. JNK plays a critical role in controlling diverse cellular functions such as cell proliferation, differentiation, and cell death in stimuli and cell-type-dependent manners.[5] It has been suggested that JNK activation is one of the main cellular events in response to various forms of RNS.[6] At present, a number of intriguing questions regarding RNS-mediated JNK activation remain to be further investigated. First, the signaling pathway controlling RNS-mediated JNK activation has not been well elucidated. It has been shown that RNS activate JNK through MAPK or extracellular signal-regulated kinase kinase (MEK) kinase 1 (MEKK1), apoptosis signal-regulating kinase (ASK1) and MAPK kinase 4 (MKK4),[7] without knowing the signaling events upstream of ASK1 or MEKK1. Second, JNK is known to play an important role in RNS-induced apoptosis.[8] It remains to be determined whether JNK is also involved in RNS-induced non-apoptotic cell death. In this study, we first identified JNK1 as the key effector molecule in RNS-induced caspase-independent non-apoptotic cell death in mouse embryonic fibroblasts (MEF). Next, we demonstrated that RNS-induced JNK1 activation and cell death requires tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and ASK1. Finally, we provided evidence

that translocation of TRAF2 and JNK to membrane lipid raft is required for RNS-mediated JNK1 activation and cell death. Taken together, data from our study reveal a novel signaling pathway controlling RNS-induced JNK activation and non-apoptotic cell death.

Results

RNS induce caspase-independent non-apoptotic cell death in MEF. RNS have been reported to induce cell death, including both apoptosis and necrosis, depending on the intensity of stimulation and the cell type tested.[2] In this study, two NO donors (sodium nitroprusside (SNP) and ($\pm$)-S-nitroso-N-acetylpenicillamine (SNAP)) and one ONOO-donor (3-morpholynosidenonimine hydrochloride (SIN-1)) were used to treat the wild-type mouse embryonic fibroblasts (WT MEF). As shown in Figure 1a and b, all the three different RNS donors induce cell death dose-dependently, as measured by either morphological changes or the cell viability test. To determine the form of cell death induced by RNS, we systematically compared the morphological and biochemical features of RNS-induced cell death with that of the typical apoptosis induced by TNFα plus Actinomycin D (ActD). It was found that SNP induced evident membrane disruption at the early stage of cell death
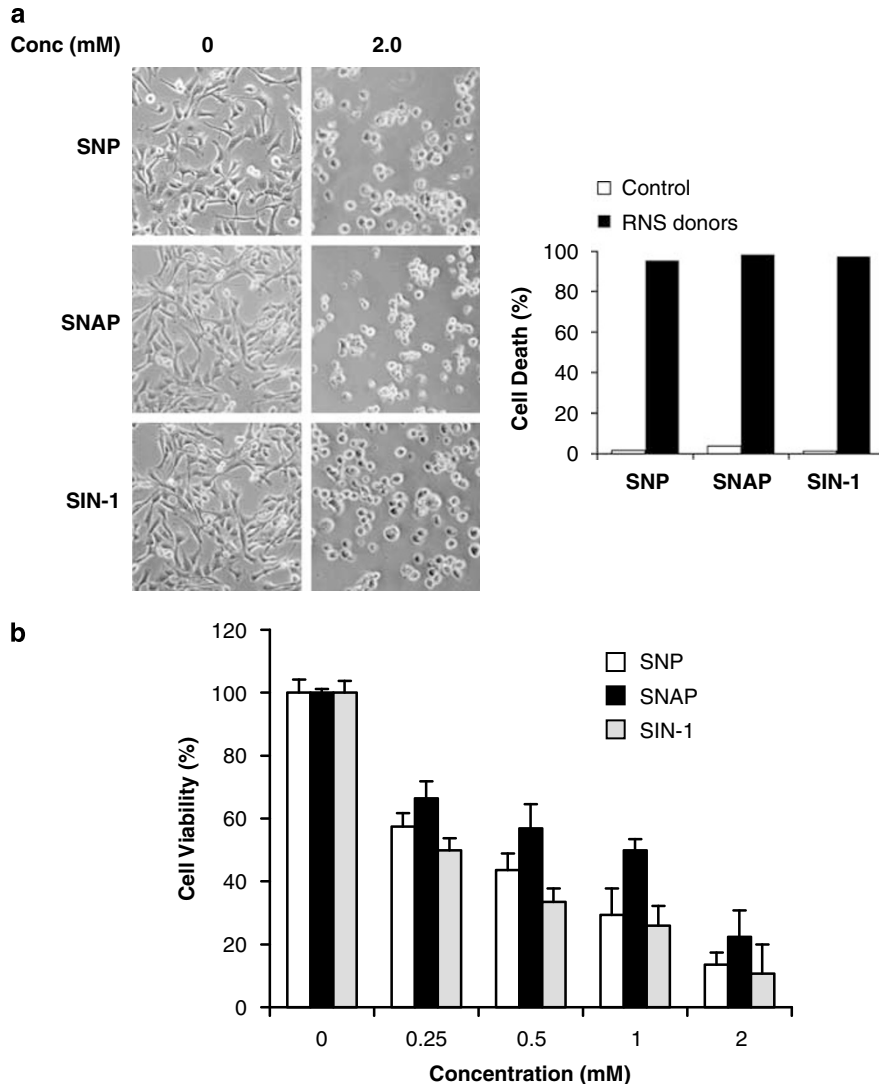
Figure 1 RNS induce caspase-independent non-apoptotic cell death in MEF. (**a**) Cell death induced by various RNS donors in WT MEF. WT MEF cells were treated with 2 mM SNP, SNAP or SIN-1 for 12 h. Representative images were taken by a phase-contrast microscope (× 200). The right panel shows the quantification of cell death based on the percentage of rounded cells in total of 200 randomly selected cells. (**b**) RNS donors-induced cell death dose-dependently. MEF were treated with various RNS donors for 12 h and the relative cell viability was quantified using MTT test. (**c**) SNP-induced cell death evaluated using Sytox–Hoechst-staining. WT MEF cells were treated with SNP (0.5 and 1.0 mM) for 6 h. ActD (5 μg/ml) plus TNFα (10 ng/ml × 6 h) was used as the positive control to induce typical apoptosis. (**d**) Absence of caspase 3 and PARP cleavage in SNP-treated cells. WT MEF cells were treated with SNP as indicated. In one group, cells were pretreated with Z-VAD-fmk (20 μM × 1 h), followed by ActD + TNFα for 6 h. Cleavage of caspase 3 and PARP was determined using Western blot. (**e**) RNS-induced cell death not affected by Z-VAD-fmk. WT MEF cells were treated with SNP for 12 h with/without pretreatment of Z-VAD-fmk (50 μM × 1 h). The cell viability was measured by MTT test. Data in (**b**) and (**e**) were presented as mean ± S.D. from at least three independent experiments

c

d

e

Figure 1 Continued.

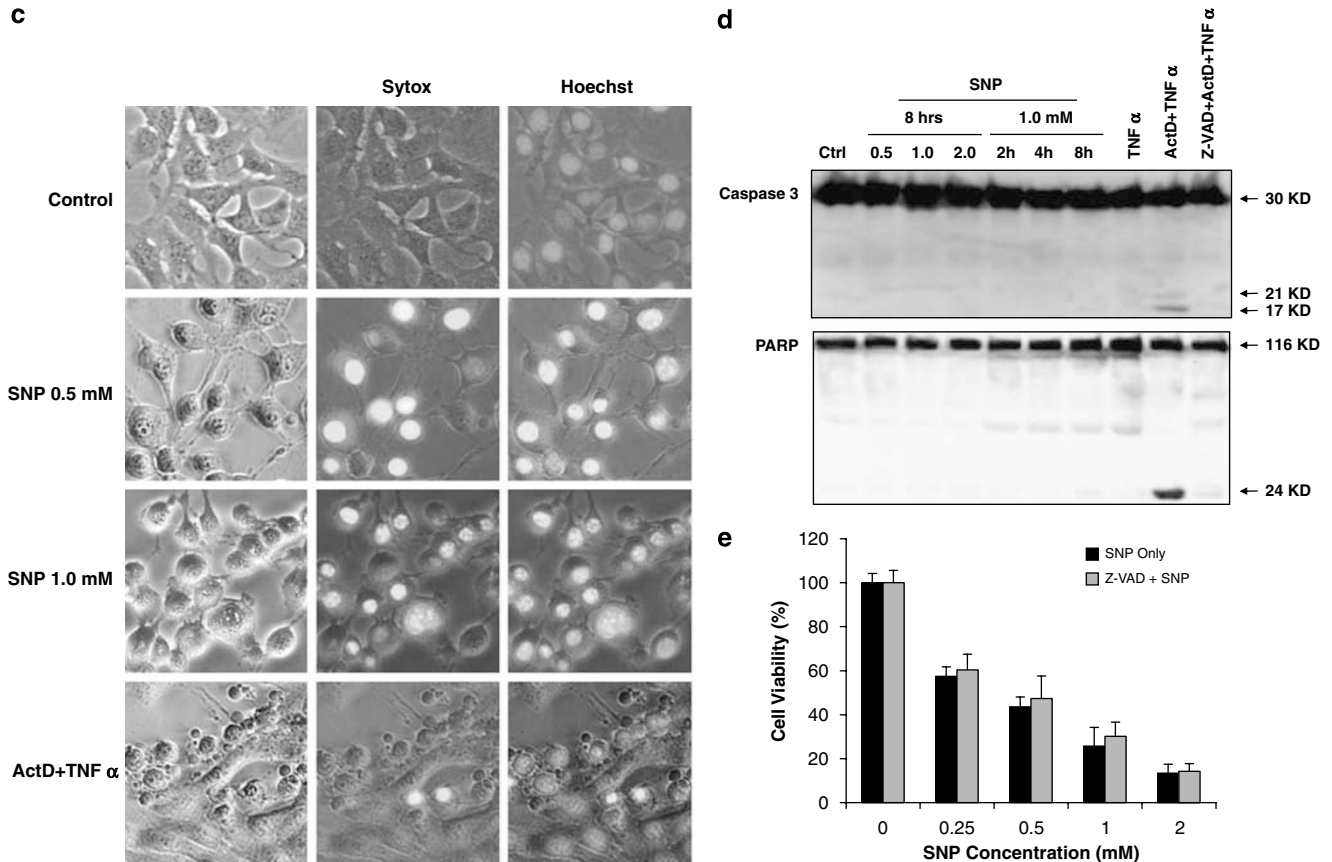indicated by Sytox staining (Figure 1c), without obvious membrane blebs and nuclear condensation indicated by Hoechst staining (Figure 1c). In contrast to typical apoptosis induced by ActD plus TNFα, no caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage was found in SNP-treated cells (Figure 1d). Moreover, SNP-induced cell death is not affected by pretreatment of Z-Val-Ala-Asp(OCH3)-Fluoromethylketone (Z-VAD-fmk) (Figure 1e), a general caspase inhibitor, which was proved to be able to abrogate caspase 3 and PARP cleavage in apoptotic MEF cells treated with ActD plus TNFα (Figure 1d). Similar results were observed in cell treated with SNAP and SIN-1 (data not shown). It is thus believed that RNS mainly induced non-apoptotic cell death independent of the caspase cascade.

JNK1 plays a critical role in RNS-induced non-apoptotic cell death. JNK has been well established as the key modulator for various stress responses, including nitrosative stress.[9] Although JNK is known to play an important role in RNS-induced apoptosis,[8,10] it remains to be determined whether JNK is also involved in RNS-induced non-apoptotic cell death. In this study, we first observed a relatively slow but sustained JNK activation after SNP treatment: evident JNK activation started from 2 h and reached the peak at 4 h (Figure 2a). Sustained JNK activation was observed up to 9 h after treatment (data not shown). SP 600125 (SP), a specific general JNK inhibitor, is able to suppress SNP-induced JNK

activation (Figure 2a) and effectively protects against SNP-induced cell death in MEF cells (Figure 2b), suggesting the critical role of JNK in RNS-mediated cell death.

Three JNK isoforms have been reported: the ubiquitously expressed JNK1 and JNK2 and the neuronal-specific JNK3.[5] To further understand the importance of JNK in RNS-induced cell death, we utilized the *jnk1−/−* MEF. As shown in Figure 2c and d, *jnk1−/−* MEF are rather resistant to cell death induced by three different RNS donors. More importantly, JNK1 protein reconstitution by stable expression of ectopic JNK1 in the *jnk1−/−* MEF (Figure 3a) effectively restored the susceptibility of *jnk1−/−* cells to SNP-induced cell death (Figure 3b and c). In addition, we also tested the *jnk2−/−* MEF and found out that *jnk2−/−* MEF responded to RNS treatment similarly as WT MEF (data not shown). Therefore, JNK1, but not JNK2, appears to play an important role in determining susceptibility of MEF to RNS-induced caspase-independent non-apoptotic cell death.

TRAF2 is required for RNS-induced JNK activation and cell death. After establishing the role of JNK1 in RNS-induced cell death, here we attempted to elucidate the signaling pathway controlling RNS-induced JNK activation. TRAF2 is one of the key signaling molecules upstream of JNK, especially in the TNF receptor 1 (TNFR1) pathway.[11] We have reported previously that TRAF2 plays an important role in reactive oxygen species (ROS)-induced JNK
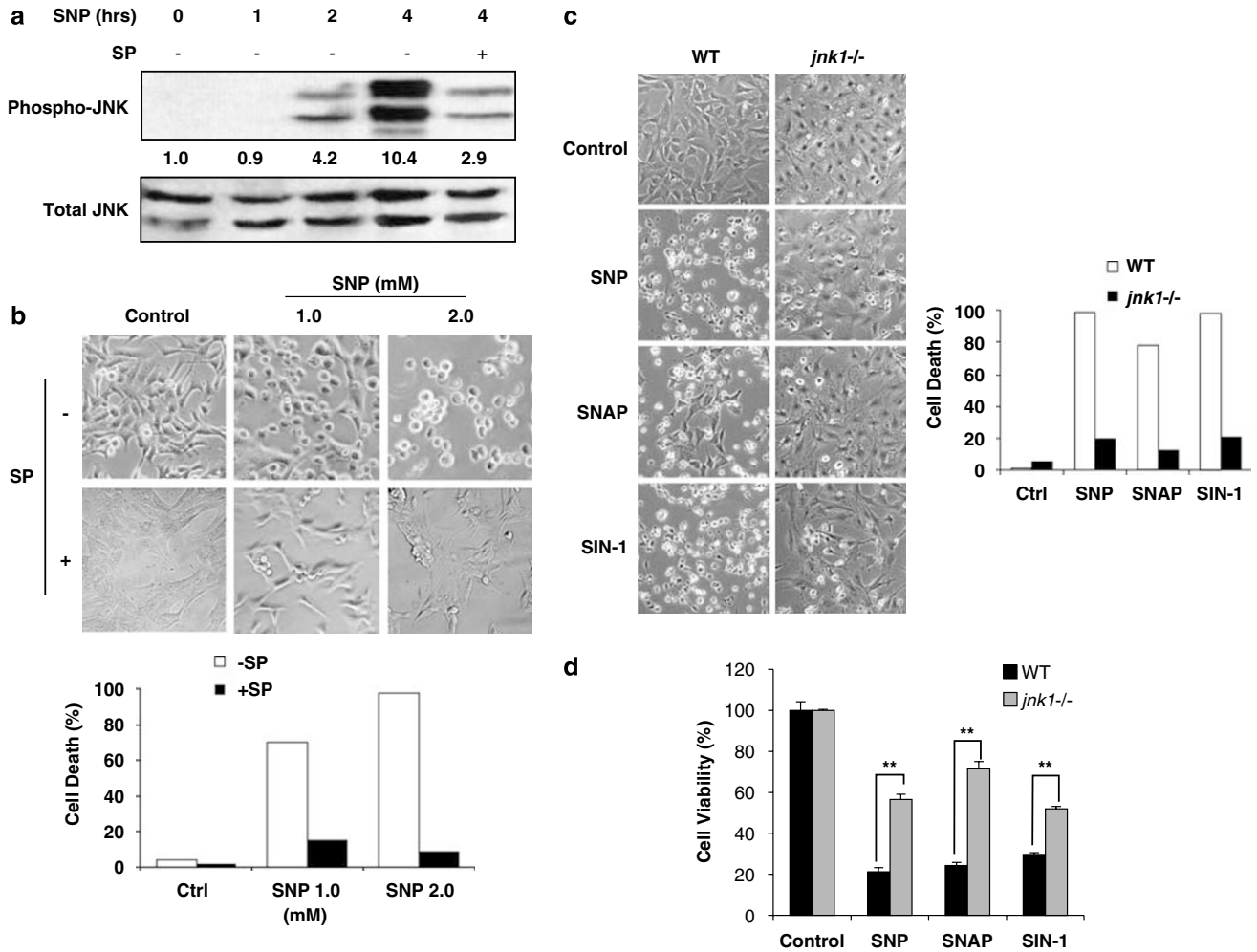
Figure 2 JNK is required for RNS-induced cell death. (**a**) SNP induces JNK activation. WT MEF cells were treated with SNP (1 mM) for designated time with/without 1 h pretreatment of JNK inhibitor SP (20 μM) and the phospho- and total JNK were detected by Western blotting. (**b**) JNK inhibitor protects WT MEF from SNP-induced cell death. WT MEF cells were treated with SNP (up to 2 mM) for 12 h with/without SP pretreatment (20 μM $\times$ 1 h). Representative images were taken by a phase-contrast microscopy showing the morphological changes of the cell ($\times$ 200). Quantification of cell death according to the numbers of rounded cells was shown in the lower panel. (**c**) jnk1$-$/$-$ MEF are resistant to RNS donors-induced cell death. WT and jnk1$-$/$-$ MEF cells were treated with SNP, SNAP and SIN-1 (at 2 mM), respectively, for 12 h. Representative images were taken by a phase-contrast microscopy ($\times$ 200). Quantification of cell death according to the numbers of rounded cells was shown in the right side panel. (**d**) MEF deficient of JNK1 are resistant to RNS donors-induced cell death. WT and jnk1$-$/$-$ MEF cells were treated as described in (**c**), and the relative cell viability was measured using MTT test and the data were presented as mean $\pm$ S.D. from three independent experiments (**$P < 0.01$, t-test)

activation and necrotic cell death.[12] Here we utilized the traf2$-$/$-$ MEF to test the relevance of TRAF2 in RNS-mediated JNK activation and cell death. As shown in Figure 4a and d, the traf2$-$/$-$ cells are almost completely resistant to cell death induced by SNP, SNAP and SIN-1 (Figure 4a and d). In contrast, the MEF deficient of receptor interacting protein 1 (RIP1) were found to respond to RNS treatment similarly to WT MEF (data not shown). Such a finding is indeed contradictory to the effect of ROS in which both RIP and TRAF2 were required for JNK activation and cell death.[12] Strikingly, RNS-induced JNK activation was completely abolished in the traf2$-$/$-$ MEF (Figure 4e), implying that (i) TRAF2 is required for RNS-mediated JNK activation and (ii) resistance of traf2$-$/$-$ MEF to RNS is due to lack of JNK activation. To further strengthen our arguments, we reconstituted TRAF2 protein into traf2$-$/$-$ cells via stable transfection (Figure 4b). The reconstitution of

TRAF2 protein completely restored the sensitivity of traf2$-$/$-$ MEF to SNP-induced JNK activation (Figure 4e) and cell death (Figure 4c and d). All these observations clearly suggest that TRAF2 is a critical regulator controlling the RNS-induced JNK activation and cell death.

ASK1 is an important mediator between TRAF2 and JNK in RNS-induced cell death. In the TNFR signaling pathway, ASK1 serves as an important signaling protein-mediating JNK activation downstream of TRAF2.[13] Role of ASK1 has also been well established for oxidative stress-induced JNK activation.[14] On the other hand, there are conflicting reports on whether RNS activate or inhibit ASK1.[7,15] Here we explored the involvement of ASK1 in RNS-induced JNK activation and cell death. In WT MEF, SNP induced rapid and transient ASK1 activation (Figure 5A), preceding RNS-induced JNK activation
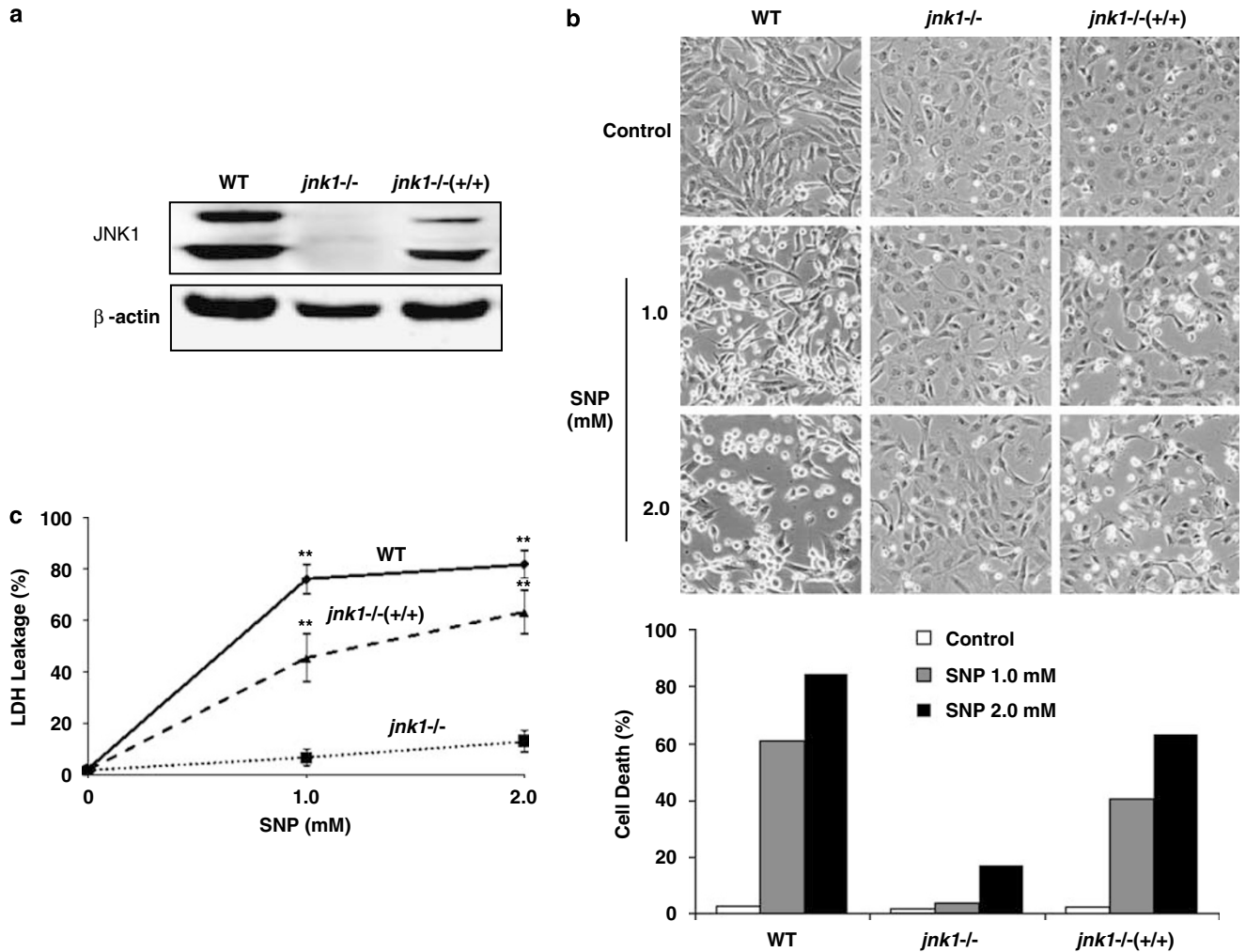
Figure 3 JNK1 reconstitution restores the susceptibility of *jnk1*−/− cells to RNS-induced cell death. (**a**) Validation of JNK1 gene knockout and stable reconstitution by Western blot. (**b**) Stable reconstitution of JNK1 protein restored the susceptibility to RNS-induced cell death. Cells were treated with SNP (up to 2 mM) for 12 h and the morphological changes were recorded using a phase-contrast microscope (×200). Quantification of cell death according to the numbers of rounded cells was shown in the lower panel. (**c**) Quantification of SNP dose-dependent cell death of WT, *jnk1*−/− and *jnk1*−/−+/+ MEF cells by LDH leakage assay. (**P*<0.01, *t*-test, comparing with the control group)

(Figure 2a). Notably, in *traf2*−/− cells, SNP failed to induce any ASK1 activation (Figure 5A), consistent with the absence of JNK activation observed earlier (Figure 4e). Moreover, stable reconstitution of TRAF2 in *traf2*−/− cells almost completely restored ASK1 activation (Figure 5A). The high background of both ASK1 and JNK activation in the TRAF2-reconstituted cells (Figures 4e and 5A) is likely due to the higher level of ectopically expressed TRAF2 protein (Figure 4b). All these observations collectively support the notion that RNS-induced JNK activation is achieved through TRAF2 and ASK1.

To further elucidate the importance of ASK1 in RNS-induced cell death, we transiently transfected WT MEF cells with a dominant-negative *ask1* (*ask1* without the functional coiled-coil domain (pcDNA-*ask1*Δcoil)),[16] together with a GFP expression vector as the transfection marker (green fluorescence indicating successful transfection). Based on the morphological changes, it is clear that cells with successful transfection of *ask1*Δcoil plasmid were found to be resistant

to SNP-induced cell death in comparison to those with successful transfection of empty pcDNA vector (Figure 5B), suggesting that RNS-induced cell death requires a functional ASK1.

RNS engage membrane lipid rafts for JNK activation and cell death. Lipid rafts are membrane microdomains enriched of glycosphingolipid and cholesterol in which lipid acyl chains are tightly packed and highly extended.[17] Lipid rafts have been proven to be important in many signaling pathways triggered by various inducers.[18] In our attempt to elucidate the signaling events upstream of TRAF2 in RNS-induced JNK activation and cell death, we explored the possible involvement of membrane lipid rafts. First, it was found that pretreatment with methyl-β-cyclodextrin (MβCD), a known lipid raft structure disruptor by depleting cholesterol,[17,19] significantly protected cells from death induced by SNP (Figure 6a and b). Next, MβCD pretreatment almost completely blocked RNS-induced JNK
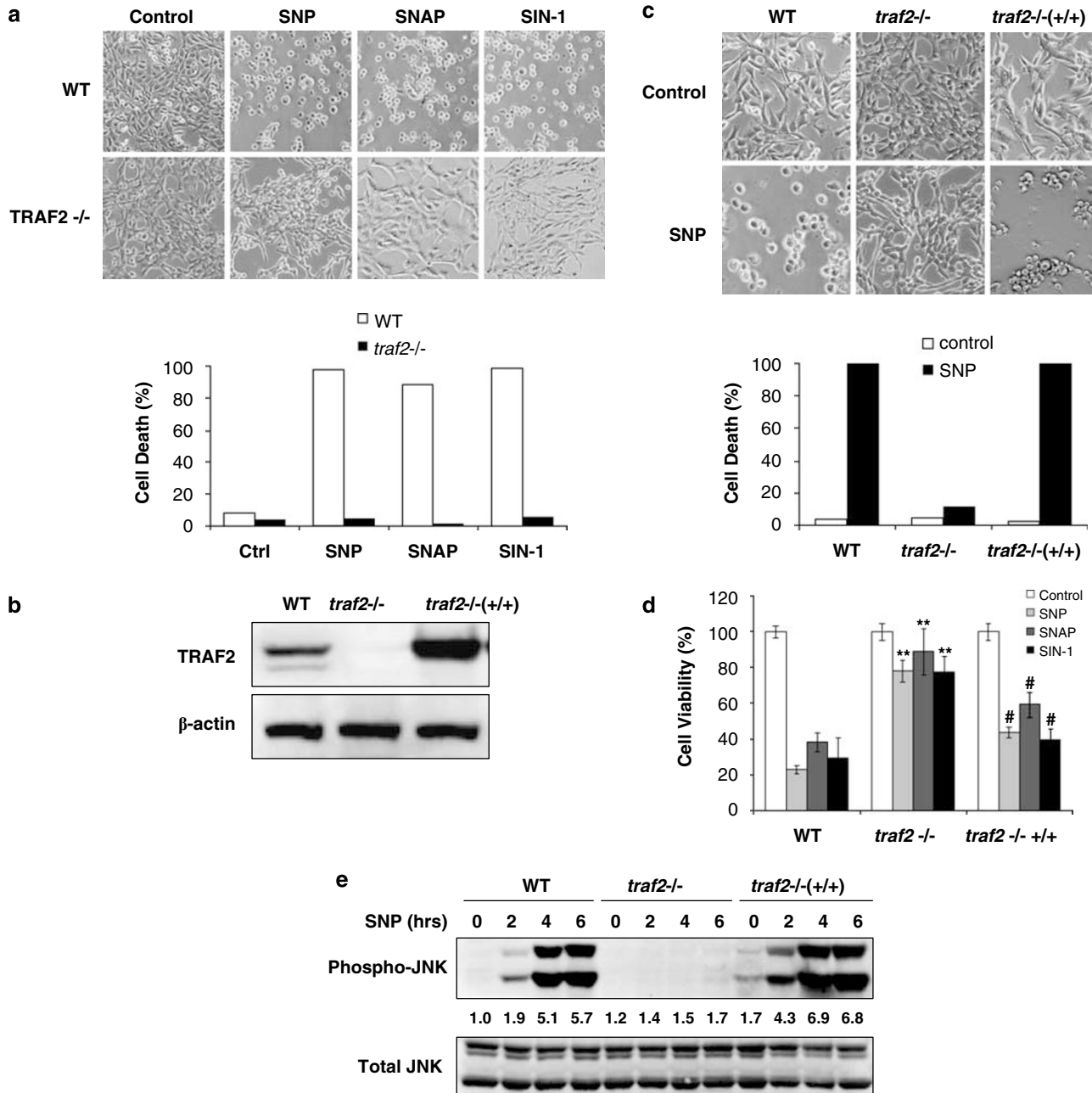
Figure 4 TRAF2 is an essential upstream regulator in RNS-induced JNK activation and cell death. (**a**) TRAF2 knockout MEF (traf2−/−) are resistant to RNS-induced cell death. WT and traf2−/− MEF cells were treated with SNP, SNAP and SIN-1 (at 1 mM), respectively, for 12 h. Representative images of cell morphological changes were taken by a phase-contrast microscope ($\times$ 200). Quantification of cell death according to the numbers of rounded cells was shown in the lower panel. (**b**) Validation of TRAF2 gene knockout and TRAF2 protein reconstitution in traf2−/− and traf2−/− (+ / +) MEF cells by Western blot. (**c**) Stable reconstitution of TRAF2 protein sensitizes traf2−/− cells to RNS-induced cell death. Representative images ($\times$ 200) were taken for the morphological changes after cells were treated with SNP (1 mM $\times$ 12 h). Quantification of cell death according to the numbers of rounded cells was shown in the lower panel. (**d**) TRAF2 is required for RNS-induced cell death. WT, traf2−/− and traf2−/− (+ / +) MEF were treated with SNP, SNAP or SIN-1 (at 1 mM) for 12 h, the cell viability was determined by MTT test and the data were presented as mean ± S.D. from three independent experiments (**$P < 0.01$, t-test, comparing with the WT cells with the same treatments, #$P < 0.05$, t-test, comparing with the traf2−/− cells with the same treatments). (**e**) TRAF2 is essential for SNP-induced JNK activation. After treatment with SNP (1.0 mM) for the designated time, cells were collected and lysed in M2 buffer and the proteins were subjected to immunoblotting using both the phospho-JNK and total JNK antibodies

activation (Figure 6c). In contrast, MβCD even markedly enhanced 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced JNK activation (Figure 6c). It is known that TPA activates JNK mainly through protein kinase C independent of lipid rafts.[20] Although it is not known how MβCD

augmented TPA-induced JNK activation, data from this part of our study clearly suggest that the inhibitory effect of MβCD on RNS-induced JNK activation and cell death is achieved through a specific mechanism, most probably via the disruption of the membrane lipid rafts. Such observations
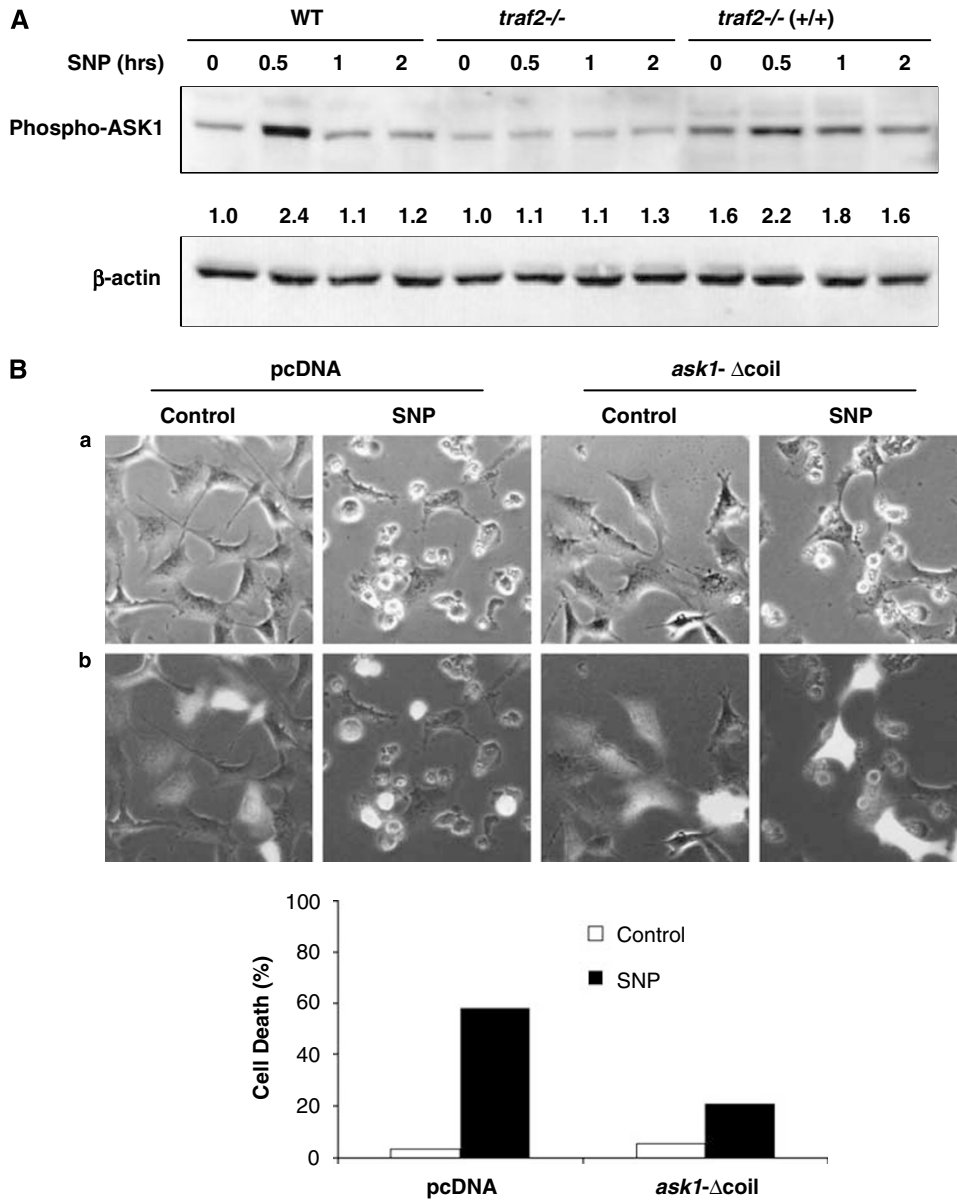
A

	WT				traf2-/-				traf2-/- (+/+)			
SNP (hrs)	0	0.5	1	2	0	0.5	1	2	0	0.5	1	2
Phospho-ASK1												
	1.0	2.4	1.1	1.2	1.0	1.1	1.1	1.3	1.6	2.2	1.8	1.6
β-actin												

B

Figure 5 ASK1 plays a critical role downstream of TRAF2 in RNS-induced JNK activation and cell death. (**A**) TRAF2 is required for SNP-induced ASK1 activation. WT, *traf2*−/− and *traf2*−/− (+/+) cells were treated with SNP (1 mM) for designated times and subjected to Western blot for phospho-ASK1. (**B**) Overexpression of mutant ASK1 suppresses SNP-induced cell death. WT MEF were transiently transfected with pcDNA3.1 or pcDNA-ASK1Δcoil, together with pMaxFP™-Green vector as the transfection marker (cells with green fluorescence were considered as with successful transfection). Cells were treated with SNP (1 mM) for 12 h, 24 h after transfection. Representative images of cell morphological changes were taken by a phase-contrast and fluorescent microscope (×400) (**a**) phase-contrast; (**b**) merged. Quantification of cell death was shown in the lower panel according to the percentage of rounded cells in a total of 100 cells with green fluorescence

also help us to rule out the possibility that MβCD directly reacts and inhibits JNK activation.

To further understand the role of lipid raft microdomains in our system, we investigated the localizations of TRAF2 and JNK within the soluble and insoluble fractions prepared by nonionic detergent which respectively represent the cyto-plasmic and cytoskeleton/membrane (including lipid rafts) fractions.[21,22] The purity of these two fractions was confirmed by RIP1 and caveolin, the marker proteins for cytoplasmic and lipid raft fractions.[22] As shown in Figure 6d, TRAF2 and JNK are all localized in soluble fractions in the control cells. With

SNP treatment, there is a time-dependent translocation of both TRAF2 and JNK to the insoluble fractions: TRAF2 translocation starts earlier (at 15 min), but less evident, followed by more significant JNK translocation (at 30 min). Since evident JNK activation in RNS-treated cells was found from 2 h onward (Figures 2a and 4e), it is thus clear that TRAF2 and JNK translocation precedes JNK activation. Moreover, pretreating cells with MβCD almost totally blocked TRAF2 and JNK translocation induced by SNP (Figure 6e), thus suggesting that the membrane lipid raft structure plays a critical role in recruiting some of the key signaling molecules

such as TRAF2, which then provides an optimal environment for JNK activation and subsequently cell death.

Discussion

JNK is the key member of the MAPK family and plays critical roles in regulating cellular responses to various stressors, including the cell death process.[5,23,24] In this study, we provide evidence demonstrating the critical role of JNK activation in RNS-induced non-apoptotic cell death. Such a finding is indeed consistent with some recent observations that JNK is an effector molecule in non-apoptotic or necrotic cell death mediated by a number of other stimuli such as ROS and TNFα.[12,14,25] On the other hand, there is evidence, suggesting the role of JNK in RNS-induced apoptotic cell death. For instance, either exogenously administered or endogenously produced RNS were capable of activating JNK and inducing apoptosis in various cell types.[26] Therefore, data from our study expand the functional scope of JNK in response to RNS and it is likely that JNK is involved in both types of cell death elicited by RNS.

The effect of RNS on JNK has been found to be double-edged: RNS may activate or inhibit JNK depending on the exposure condition and cell type. The inhibitory effect of RNS on JNK is based on their direct modification of JNK protein by means of S-nitrosylation.[27] For example, Park *et al.*[28] have identified cysteine 116 as the site of such reaction in JNK1. RNS may also inhibit ASK1 activity via a similar mechanism, leading to inhibition of JNK activation.[15] On the other hand, RNS activate JNK via the typical MAPK activation pathway involving the MAPK kinase kinase (MAPKKK) and MAPK kinase (MAPKK) as upstream kinases. Two MAPKKK (MEKK1 and ASK1) and one MAPKK (MKK4) have been implicated in RNS-induced JNK activation.[7,29,30] However, little is known about the signaling mechanism upstream of MAPKKK in RNS-mediated JNK activation. In this study, we provide unequivocal evidence showing the critical role of TRAF2 upstream of ASK1 in RNS-mediated JNK activation.

TRAF2 is a key signaling molecule in TNF-mediated JNK activation[11,31] and TRAF2 is known to interact directly with ASK1 to activate JNK.[13,32] Moreover, TRAF2 has been shown to be involved in ROS-induced JNK activation and cell death.[12,14] In this study, we demonstrate, for the first time, that TRAF2 is required for RNS-induced JNK activation and non-apoptotic cell death, based on the following observations: (i) RNS failed to induce JNK activation and cell death in *traf2−/−* MEF (Figure 4a, d and e) and (ii) TRAF2 reconstitution restored both responses (Figure 4c–e). As discussed above, ASK1 has been shown to be involved in RNS-induced JNK activation and apoptosis.[7] Recently, ASK1 has also been reported to be involved in regulation of ROS-induced necrotic cell death.[33] For instance, in cells treated with H_2O_2, the recruitment of TRAF2 to ASK1 to form a signalosome is a critical step in H_2O_2-induced cell death.[14] In the present study, RNS fail to activate ASK1 in *traf2−/−* cells (Figure 5A) and overexpression of a dominant-negative form of ASK1 protects WT MEF from RNS-induced cell death (Figure 5B). Although the direct interaction between TRAF2 and ASK1 was not measured in this study, these observations strongly suggest that ASK1 is an important molecule relaying the cell death

signal of RNS from TRAF2 to JNK. Downstream of ASK1, MKK4 or MKK7 is the two main MAPKKs for JNK activation.[5] Although we were unable to determine RNS-induced MKK4/7 activation due to the lack of suitable antibodies, overexpression of a mutant form MKK7–JNK1 fusion protein protects against RNS-induced cell death (data not shown), suggesting a possible role of MKK7 downstream of ASK1 in RNS-induced JNK activation and cell death.

We next examined the signaling mechanisms upstream of TRAF2 in RNS-induced JNK activation and cell death. Although TRAF2 is one of the key molecules in TNFR1 signaling pathway,[11,31] it appears that RNS acts on TRAF2 independent of TNFR1, based on our preliminary observation that *tnfr1−/−* MEF are as susceptible as WT MEF to RNS-induced cell death (data not shown). Lipid rafts, the liquid-ordered phase microdomains existing within membranes, have been reported involved in various signaling pathways triggered by numbers of stimuli, such as H_2O_2,[12,34] ultraviolet light (UV)[35] and TNFα.[36] Data from our study clearly support the notion that RNS promote the recruitment of TRAF2 into lipid rafts and then mediates JNK activation and subsequent cell death. Recruitment of TRAF2 into lipid rafts has been found to be crucial for TNFα-induced NF-κB and JNK activation in HT1080 and HeLa cells.[22,36] However, a recent report showed that lipid rafts are essential for TNFα-mediated activation of RhoA but dispensable for the activation of the NF-κB and MAPK pathways in human airway smooth muscle cells,[37] suggesting that the involvement of lipid rafts and TRAF2 in cell signaling is likely to be cell type specific. An intriguing question to be further studied is the mechanism for recruitment of TRAF2 into lipid rafts. A valuable clue provided by Habelhah *et al.*[22] is that the RING domain and zinc finger of TRAF2 is probably essential for its translocation to lipid raft. On the other hand, it is interesting to note that RNS treatment failed to affect RIP1 translocation (Figure 6d). RIP1 is one of the key adaptor molecules in the TNFR1 signaling and works closely with TRAF2 for subsequent signaling events, such as activation of NF-κB and JNK.[36,37] In this study, it appears that RNS specifically target TRAF2 without engaging RIP1. Such a notion is indeed supported by the fact that *rip1−/−* cells are as sensitive as WT MEF to RNS-induced cell death (data not shown). Such a selectivity on TRAF2 by RNS is also found to be different from the effect of H_2O_2 of which both TRAF2 and RIP1 are required for JNK activation and cell death.[12] At present, mechanisms for such specificity remain to be further elucidated. Moreover, it is still not clear how TRAF2 engages the downstream signaling molecules such as ASK1 and MKK4/7 at the site of lipid rafts.

Another important finding of this study is the evident JNK translocation to the insoluble fraction, following TRAF2 translocation temporally (Figure 6d). Such observations are consistent with recent findings that translocation of JNK toward lipid raft microdomains occurred when U937 cell was treated with UV-C.[35] In addition, redistribution of MAP kinases, including extracellular signal-regulated kinase and p38, to lipid raft structures in stimulated neutrophils has also been reported,[38] suggesting that membrane lipid rafts may play a role in action of the MAPK family, depending on the study context, such as cell type and the nature of the stimulus.
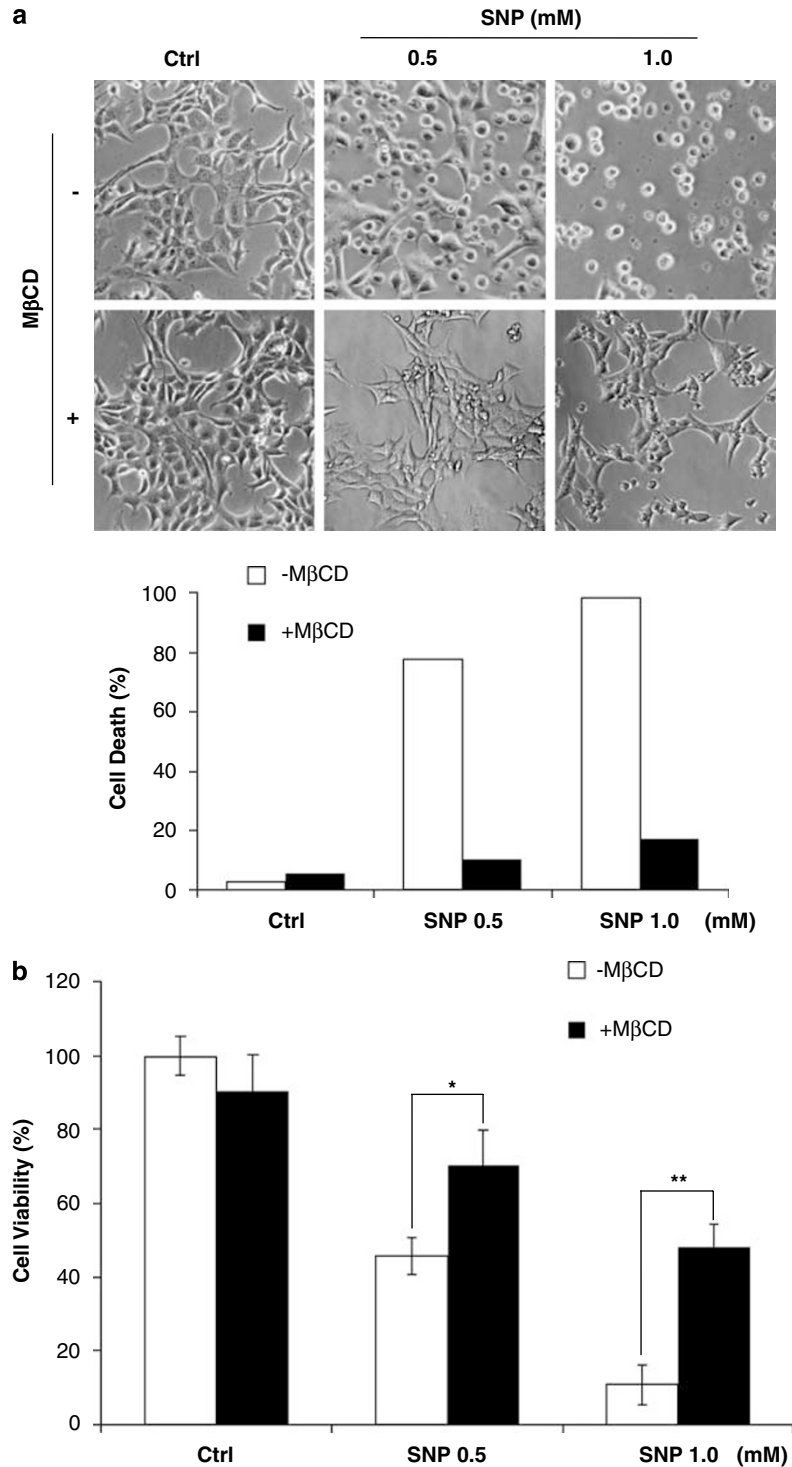
Figure 6 Lipid raft structure is critical for RNS-mediated JNK activation and cell death in MEF cells. (**a**) MβCD protects against SNP-induced cell death. WT MEF cells were pretreated with MβCD (2 mM) for 30 min, followed by designated SNP treatments for 12 h. Representative images of cell morphological changes were taken by a phase-contrast microscope ($\times$ 200). Quantification of cell death according to the numbers of rounded cells was shown in the lower panel. (**b**) Quantification of cell viability upon SNP treatment with/without pretreatment of MβCD by MTT test. WT cells were treated as described in (**a**), and the data were presented as mean $\pm$ S.D. from three independent experiments (*$P < 0.05$, **$P < 0.01$, *t*-test). (**c**) MβCD blocks the phosphorylation of JNK. WT MEF cells were pretreated with MβCD (2 mM) for 30 min, followed with SNP (1.0 mM $\times$ 3 h) and TPA (80 nM $\times$ 1 h), respectively. (**d**) TRAF2 and JNK translocation. WT MEF cells were treated with SNP (1.0 mM) for designated time and cell fractionation was performed as described in Materials and Methods. Phospho-JNK, total-JNK and TRAF2 were analyzed by Western blot. RIP and caveolin-1 were used as markers for the cytoplasmic and raft fractions, respectively. (**e**) MβCD blocks the translocations of JNK and TRAF2. WT MEF cells were pretreated with MβCD (2.0 mM) for 30 min, followed by SNP (1.0 mM) treatments for 2 h. The insoluble fraction was subjected to immunoblotting as described in (**d**)

c

SNP (1.0 mM)	-	+	-	+	-	-
MβCD (2.0 mM)	-	-	+	+	-	+
TPA (80 nM)	-	-	-	-	+	+

Phospho-JNK

| | 1.0 | 4.1 | 1.2 | 1.8 | 2.0 | 4.2 |

Total-JNK

d

| | Soluble fractions | | | | | | Insoluble fractions | | | | | |
| SNP (1.0 mM) | Ctrl | 5 | 15 | 30 | 60 | 120 | Ctrl | 5 | 15 | 30 | 60 | 120 | min |

Total-JNK

| | | | | | | | 1.0 | 0.6 | 0.8 | 2.5 | 5.6 | 6.3 |

TRAF2

| | | | | | | | 1.0 | 0.9 | 1.3 | 1.3 | 1.8 | 1.2 |

RIP

| | | | | | | | 1.0 | 0.8 | 0.6 | 0.6 | 0.9 | 0.7 |

Caveolin-1

| | | | | | | | 1.0 | 1.0 | 1.1 | 1.2 | 1.3 | 1.2 |

e

	Insoluble fraction			
SNP (1.0 mM)	-	-	+	+
MβCD (2.0 mM)	-	+	-	+

Total-JNK

| | 1.0 | 1.2 | 3.3 | 0.8 |

TRAF2

| | 1.0 | 1.3 | 2.1 | 1.4 |

RIP

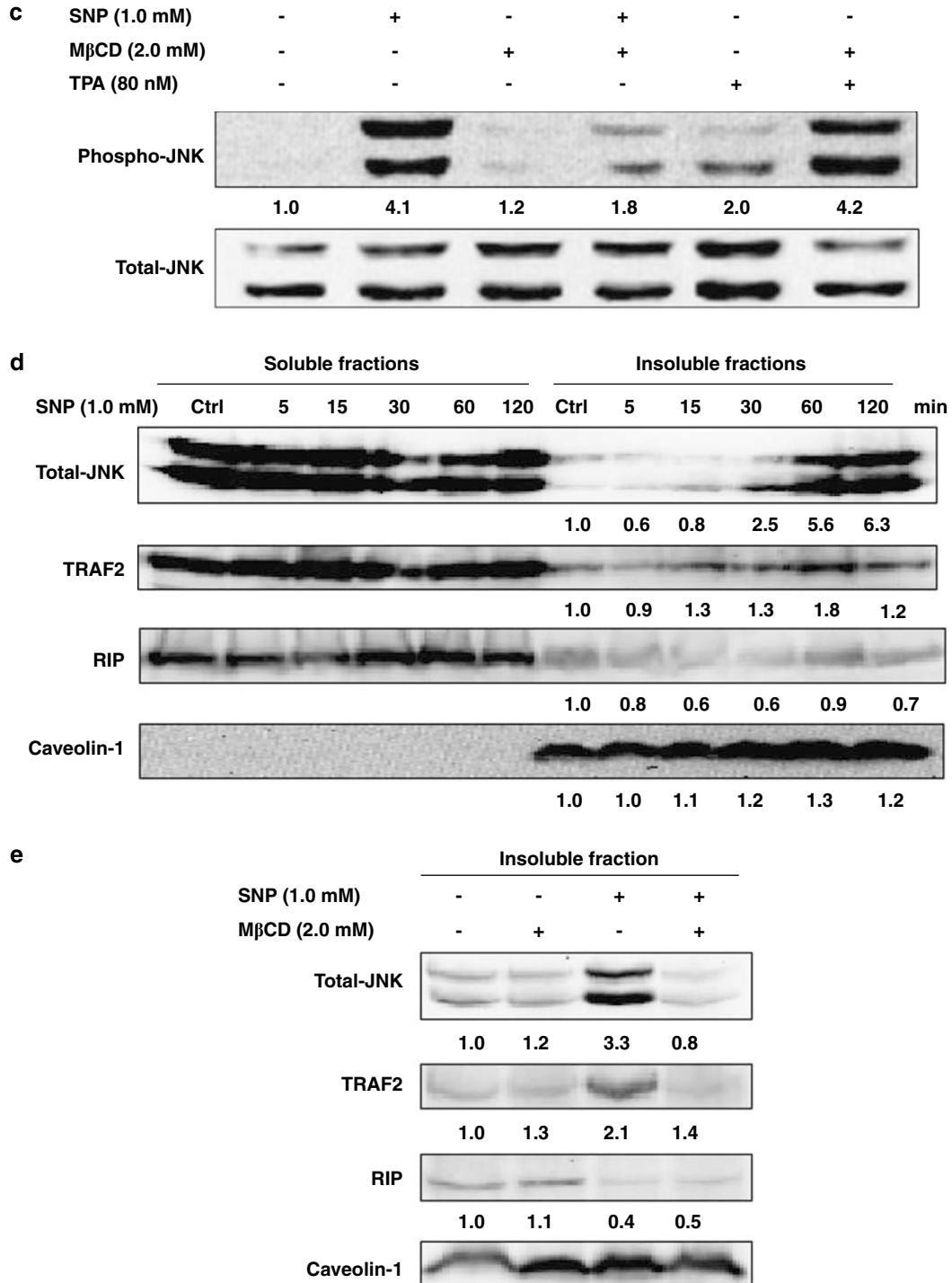| | 1.0 | 1.1 | 0.4 | 0.5 |

Caveolin-1

Figure 6 Continued.

Apoptosis and non-apoptotic cell death/necrosis are two distinct forms of cell death and both are closely implicated in the pathological processes of human diseases.[39] Recent accumulating evidence has given rise to the idea that non-apoptotic or necrotic cell death is executed via defined machinery without the caspase activation and typical apoptotic morphological features.[40,41] In this work, RNS are found to induce caspase-independent non-apoptotic cell death via a signaling pathway involving lipid raft, TRAF2-ASK1-JNK cascade. Knowledge of such a novel signaling pathway by RNS certainly helps to understand the pathological role of RNS and nitrosative stress in human diseases.

Materials and Methods

Reagents and antibodies. RNS donors (SNP, SIN-1 and SNAP) and JNK inhibitor (SP) were purchased from Calbiochem (San Diego, CA, USA).

Pan-caspase inhibitor Z-VAD-FMK was purchased from BioMol (Plymouth meeting, PA, USA). Hoechst 33342 and Sytox were purchased from Invitrogen (Molecular Probes, Eugene, OR, USA). Anti-phospho-JNK (Thr[183]/Tyr[185]) and anti-caspase3 antibodies were from Cell Signaling (Beverly, MA, USA). Anti-TRAF2 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); anti-JNK1 (clone G151-333), anti-JNK (clone G151-666), anti-caveolin, anti-PARP and anti-RIP antibodies were purchased from BD Pharmingen (San Diego, CA, USA). MβCD, anti-β-actin antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-phospho-ASK-1 antibody has been described previously.[16]

Cell culture and treatments. MEF cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (Invitrogen) in a 5% CO_2 atmosphere at 37°C. SNP (dissolved in FBS-free medium), SIN-1 (dissolved in pH 5.0 sodium phosphate buffer) and SNAP (dissolved in DMSO) were prepared as stock solution (500 mM) immediately before treatment.

Detection of cell death. Cell death induced by RNS donors was quantified by using the following three methods: (i) 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) test as described previously and the results were presented as the relative cell viability comparing to the control group;[42] (ii) lactate dehydrogenase (LDH) leakage quantification test to examine the plasma membrane permeability, by using the cytotoxicity detection kit (Roche) or (iii) cell morphological changes, and the percentage of rounded cells counted among 200 randomly selected cells and the quantification data were presented side by side with the morphological images. For Sytox–Hoechst staining of cell, treated cells were stained with Sytox (50 nM)–Hoechst (10 μg/ml) mixture solution (in PBS) at room temperature for 30 min. The staining of nuclei was visualized and captured under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S).

Plasmids and transient transfection. pcDNA-ASK1Δcoil vector has been described previously.[16] pcDNA3.1 vector was from Invitrogen (Carlsbad, CA, USA). pMaxFP™-Green vector was from Amaxa GmbH (Koeln, Germany). The transient transfections were performed using electroporation-based Nucleofector®II device (Program A23) with MEF2 nucleofector kit (Amaxa GmbH, Koeln, Germany). The transfected cells (with green fluorescence) were examined under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S).

Cell fractionation. Separation of detergent-resistant lipid raft from detergent-soluble fractions was conducted according to previously described with some modifications.[22] Briefly, cells were washed twice in ice-cold PBS after treatments and were scraped in PBS and spun down at 2000 r.p.m. 4°C in a tabletop centrifuge (Eppendorf). The cells were subsequently lysed in TNE (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 3 mM EDTA and Roche protease inhibitor cocktail), with 1% Brij 98 at 37°C for 30 min. Afterward, the lysates were homogenized by passage through a 23-gauge needle for 10 times on ice, and then were centrifuged at 1000 *g* at 4°C for 5 min. The postnuclear supernatants were additionally centrifuged at 16 000 *g* at 4°C for 20 min. The supernatants were collected as the soluble fractions. The detergent-insoluble pellets were resuspended and briefly sonicated in the same lysis buffer supplemented with 0.5% SDS and 2 mmol/l DTT and served as the lipid raft fractions.

Western blotting. At the end of designated treatments, cells were lysed in M2 lysis buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM glycerol phosphate, 1 mM sodium vanadate and proteinase inhibitor cocktail). Equal amount of protein was fractionated on SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). After blocked with 5% non-fat milk, the membrane was probed with designated first and second antibodies and developed with enhanced chemiluminescence method (Pierce) and visualized by Kodak Image Station 440CF (Kodak). The band density was quantified using ImageJ image processing program developed by NIH and normalized to that of the control group.

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