# Prevention of TNF-induced necrotic cell death by rottlerin through a Nox1 NADPH oxidase

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## Abstract

**Previous studies have demonstrated that rottlerin, a specific PKCδ inhibitor, potentiates death receptor-mediated apoptosis through a cytochrome c-dependent or -independent pathway. However, its ability to regulate necrotic cell death, as well as the underlying mechanism, remains unknown. We found that in murine fibrosarcoma L929 cells, treatment with rottlerin protected the cells against TNF-induced necrosis, whereas it sensitized the cells to apoptosis induced by co-treatment with Hsp90 inhibitor geldanamycin and TNF, in a manner independent of its ability to inhibit PKC-δ. TNF treatment induced rapid accumulation of mitochondrial superoxide ($O_2^-$) through the Nox1 NADPH oxidase when cells undergo necrosis. Moreover, pretreatment with rottlerin failed to induce the GTP-bound form of small GTPase Rac1 by TNF treatment, and subsequently suppressed mitochondrial $O_2^-$ production and poly(ADP-ribose) polymerase activation, thus inhibiting necrotic cell death. Therefore, our**

**study suggests that Nox1 NADPH oxidase is a new molecular target for anti-necrotic activity of rottlerin upon death-receptor ligation.**

**Keywords:** cell death; necrosis; NADPH oxidase 1; rottlerin; superoxidase; tumor necrosis factor-α

## Introduction

TNF is a pleiotropic cytokine that mediates diverse biological responses ranging from inflammation to cell death (Fas et al., 2006). Although the mode of cell death induced by TNF appears to be apoptotic in most cellular systems, induction of necrosis, of the type characterized as necrotic programmed cell death, has also been observed in some types of cells, with or without signaling molecules, including caspases (Fiers et al., 1999). This indicates that, depending on the cellular context, the same stimulus can induce either apoptosis or necrosis. Recent findings have suggested that necrotic cell death occurs not only during pathological processes, but also during normal physiological processes such as tissue renewal, embryogenesis and immune responses (Mayhew et al., 1999; Murdoch et al., 1999). Importantly, despite numerous efforts, there has been little progress in increasing the efficiency of anticancer therapy by induction of apoptosis in tumor cells. The rate of apoptosis shows little correlation with suppression of clonogenic ability of cancer cells, which results in tumor recurrence. Some chemotherapeutic agents and ionizing radiation (IR) used in cancer therapy, along with apoptosis, can cause necrosis (Maurer et al., 1999; Olive et al., 1999). Therefore, further elucidation of pathways towards necrosis may not only uncover the fundamental mechanism of intracellular death signaling, but also provide a prospective strategy to increase efficiency of anticancer therapy.

Rottlerin has been used as a PKCδ-selective inhibitor in many different cellular systems. Subsequently, the alteration of biological events by rottlerin has been interpreted as a positive indication for the involvement of PKCδ in these events (Gschwendt et al., 1994). Several recent studies however, have implied that the effect of rottlerin is not caused by the direct inhibition of PKCδ. In one of these studies, rottlerin was reported to inhibit

mitochondrial metabolism, which correlated with depletion of cellular ATP concentration and general inhibition of cellular processes (Soltoff, 2001). Of note, these recent observations have implicated the apoptosis-inducing effect of rottlerin on tumor cells, and synergistic sensitization of the cytotoxic effects of chemotherapeutic and apoptotic ligands (Tillman *et al*., 2003; Kurosu *et al*., 2007).

Although accumulating evidence suggests that rottlerin plays a PKCδ-independent role in modulating apoptotic cell death signaling, its effect or underlying mechanism in necrotic cell death has never been investigated. In this study, we showed that rottlerin effectively protected murine fibrosarcoma L929 cells against TNF-induced necrosis, whereas it sensitized the cells to apoptosis induced by co-treatment with Hsp90 inhibitor geldanamycin (GA) and TNF. It is of importance that rottlerin also inhibited mitochondrial superoxide ($O_2^-$) production, through suppressing Nox1 NADPH oxidase activity which led to the inhibition of TNF-induced necrosis.

## Materials and Methods

### Cell culture and infection of recombinant PKC adenovirus

Murine fibrosarcoma L929 cells and mouse embryonic fibroblast (MEF) cells were cultured in DMEM with 10% FBS, 2 mM glutamine, 100 U/ml penicillin/streptomycin. Adenovirus expression vectors for wild type and the dominant-negative type of PKCδ have been described previously (Ohba *et al*., 1998).

### Determination of cell death

After cells were treated with the concentrations of reagents as described in the figure legends, cell death was quantified by trypan blue exclusion assay. For measurement of early apoptotic or necrotic/late apoptotic cell death, cells were stained with 10 μM FITC-labeled annexin V and PI in a $Ca^{2+}$-enriched buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and analyzed by flow cytometry. Annexin V and PI emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer, using emission filters of 488 and 532 nm, respectively.

### Western blot analysis

Upon treatment, cells were lysed in M2 buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin). For Western blotting, 50 μg protein was visualized by enhanced chemiluminescence (ECL; Amersham).

### Determination of mitochondria-derived $O_2^-$ production

Production of mitochondria-derived $O_2^-$ was measured using the $O_2^-$-sensitive, hydroethidine analog, mitochondria-targeted probe, MitoSOX Red (Molecular Probes). Briefly, cells were incubated with MitoSOX Red (5 μM) for 20 min in KH buffer (pH 7.3, 15 mM $NaHCO_3$, 5 mM KCl, 120 mM NaCl, 0.7 mM $Na_2HPO_4/NaH_2PO_4$), and the red fluorescence of ox-MitoSOX was imaged using an inverted laser scanning confocal microscope (LSM-510 META; Carl Zeiss) at λex 543 nm and λem 580 nm.

### Cellular fractionation and NADPH oxidase activity

Upon treatment, cells were lysed in isotonic buffer (10 mM HEPES/KOH, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), and subjected to centrifugation at 3,500 *g* for 5 min to obtain a post-nuclear supernatant. The mitochondrial and plasma membrane fractions were obtained by centrifugation at 19,500 *g* for 40 min and 100,000 *g* for 60 min, respectively. The extracts from fractionated samples were subjected to NADPH oxidase activity, as described (Kim *et al*., 2007b).

### Rac1 activation assay

The endogenous GTP-bound form of Rac1 was measured by Rac/CDC42 assay kit (Upstate Biotechnology), according to the manufacturer's instructions. Briefly, cells were harvested using 500 μl of lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 20 μg/ml leuptin). Cell lysates were centrifuged for 5 min at $3,000 \times g$, and the resulting supernatant were mixed with 100 μM of GTPγS and 10 μg of GST-PAK1-(67-150) conjugated to glutathione-agarose beads at 4°C for 1 h. Beads were washed four times with lysis buffer and subjected to Western blotting. Rac1-GTP was visualized using an anti-Rac1 monoclonal antibody supplied with the kit (Upstate Biotechnology).

### Statistical analysis

Data are expressed as the mean $\pm$ SE from at least three separate experiments performed in triplicate. Differences between groups were analyzed

using Student's *t* test, and *P* < 0.05 was considered statistically significant, by using SPSS software (ver. 11.0).

## Results and Discussion

### Rottlerin protects murine fibrosarcoma L929 cells against TNF-induced necrotic cell death in a PKCδ-independent manner

To explore the effect of rottlerin on TNF-induced
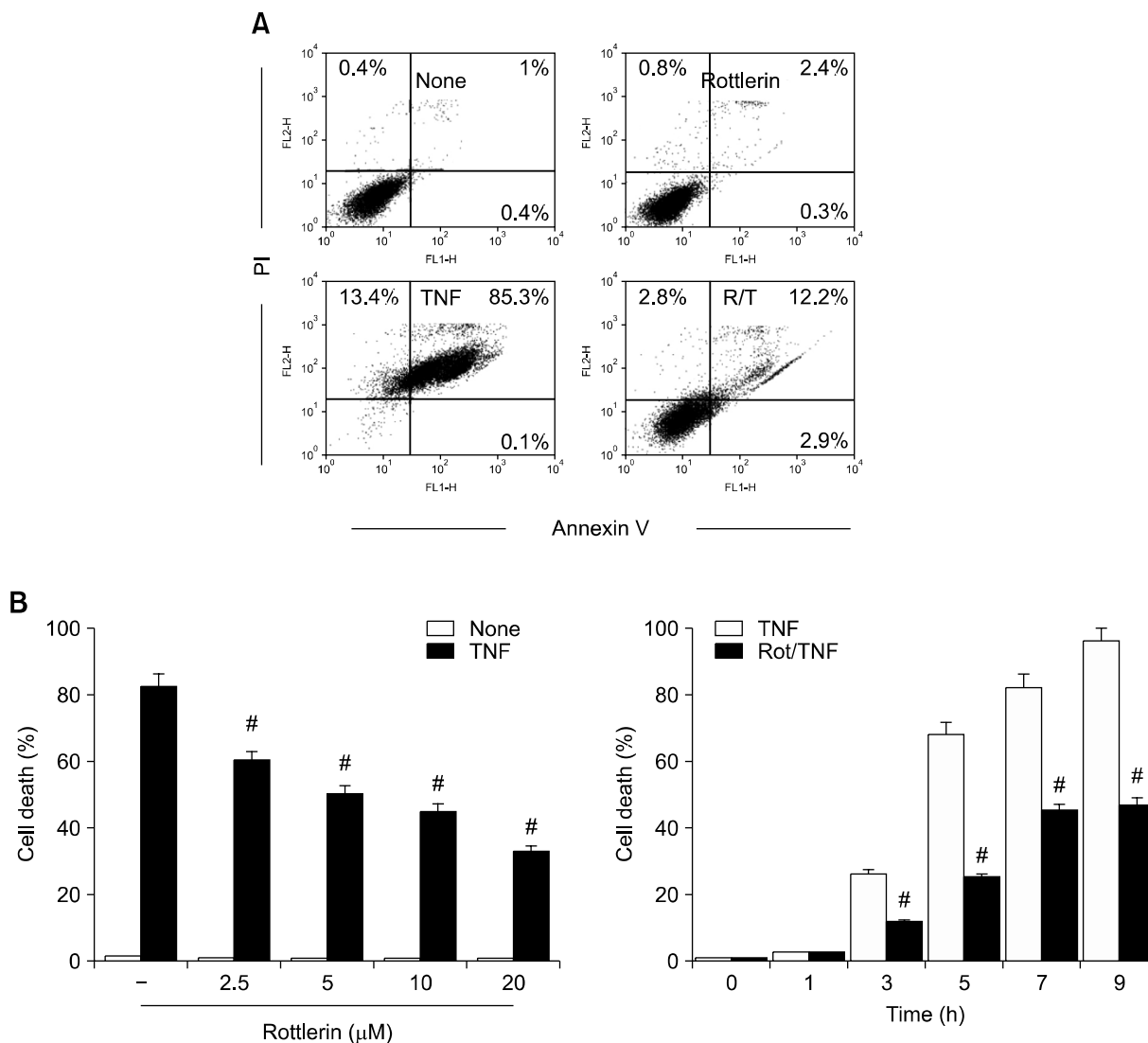


**Figure 1.** Rottlerin protects murine fibrosarcoma L929 cells against TNF-induced necrotic cell death in a PKC-δ-independent manner. (A) L929 cells were treated with 15 ng/ml TNF for 8 h in the presence or absence of 10 μM rottlerin, stained with FITC-labeled annexin V and PI, and analyzed by flow cytometry. (B) L929 cells were pretreated with rottlerin at the indicated concentrations (left) and times (right), and further treated with TNF for 8 h. Cell death was quantified by trypan blue exclusion assay. Each bar shows the mean ± SE of at least three independent experiments. [#]$P$ < 0.05 compared with TNF-treated group. (C) After pretreatment with 10 μM rottlerin, 100 μM BHA and 10 mM NAC, L929 cells were treated with 500 μM $H_2O_2$ or 10 μM menadione (MD) for 12 h, and cell death was quantified as described in (B). *$P$ < 0.05 compared with the $H_2O_2$-treated group. [#]$P$ < 0.05 compared with the MD-treated group. (D) L929 cells were infected with wild type or dominant-negative type of PKC-δ adenovirus at an m.o.i. of 100. After 48 h incubation, cells were treated with TNF (15 ng/ml) for 8 h in the presence or absence of 10 μM rottlerin, and cell death was quantified as described in B. [#]$P$ < 0.05 compared with the TNF-treated group. PKCδ activities in L929 cells infected with DN-PKCδ AdV or WT-PKCδ AdV were analyzed by immune complex kinase assay with [γ-$^{32}$P] ATP and MBP as a substrate. Phosphorylation of MBP was assessed by SDS-PAGE and autoradiography. PKCδ or actin protein content was analyzed by Western blotting with anti-PKCδ or actin antibody. (E) After pretreatment with 50 nM PMA, 10 μM Bis1, 10 μM Go6983 or 10 μM rottlerin, L929 cells were treated with TNF (15 ng/ml) for 8 h, and cell death was quantified as described in (B). [#]$P$ < 0.05 compared with the TNF-treated group.
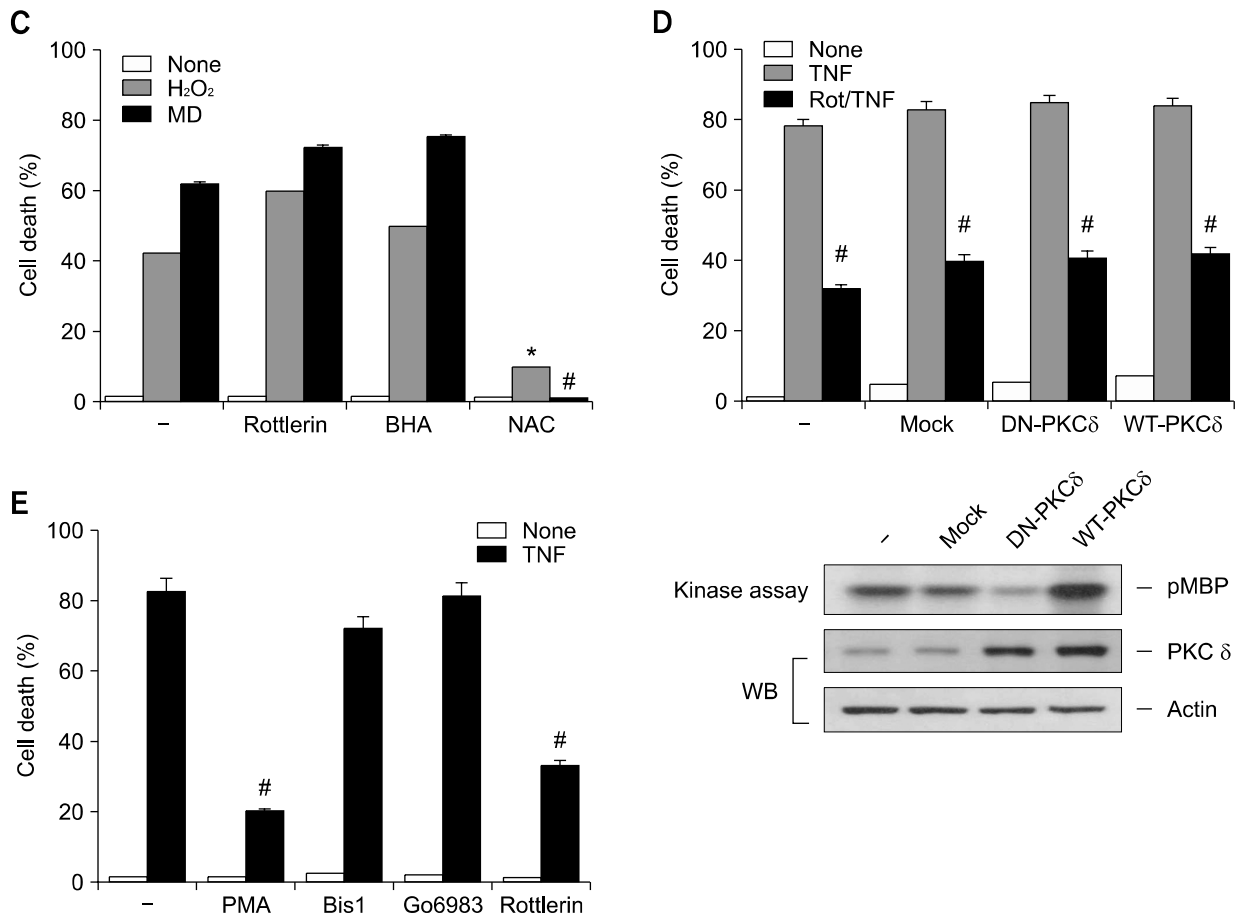
C



D



E



**Figure 1.** Continued.

necrosis, we decided to use murine fibrosarcoma L929 cells because of their known sensitivity to the necrotic action of TNF (Hehner *et al.*, 1998). Therefore, they have been widely used to investigate the mechanism of necrotic signaling. First, we tested whether TNF induced non-apoptotic cell death. As expected, treatment of cells with TNF increased the number of necrotic and late-phase apoptotic cells, whereas very few cells were stained exclusively with annexin V (Figure 1A). We next investigated the effect of rottlerin on TNF-induced necrosis. Pretreatment of L929 cells with rottlerin drastically abrogated TNF-induced necrosis in a dose- and time-dependent manner, as measured by flow cytometry and trypan blue exclusion assays (Figure 1A and B). Based on the ability of rottlerin to protect against TNF-induced necrosis, we evaluated whether it had a protective role against cell death caused by reactive oxygen species (ROS), such as $H_2O_2$ and menadione, which is caspase-independent (Sata *et al.*, 1997; Troyano *et al.*, 2003). Interestingly, pretreatment

with rottelrin did not block necrosis, whereas the antioxidant N-acetlycystein clearly protected cells against cell death caused by $H_2O_2$ and menadione (Figure 1C). More importantly, pretreatment with butylated hydroxyanisole (BHA) a well known ROS scavenger that can enter mitochondria and inhibit TNF-induced necrosis (Vercammen *et al.,* 1998), also did not block these exogenous sources of ROS-induced cell death (Figure 1C). These results suggest that, similar to BHA, rottlerin negatively and specifically regulates necrosis through the suppression of newly generated ROS in the mitochondria, upon death-receptor ligation.

Results of recent studies have demonstrated that rottlerin sensitizes some cancer cells to death-receptor- or chemotherapeutic-agent-induced apoptosis in a manner independent of PKCδ activity (Tillman *et al.*, 2003; Kurosu *et al.*, 2007). To examine the possible involvement of PKCδ in the prevention of TNF-induced necrosis by rottlerin, we introduced adenovirus expressing wild-type (WT) and dominant-negative (DN) forms of PKCδ into

**A**



**B**



**C**



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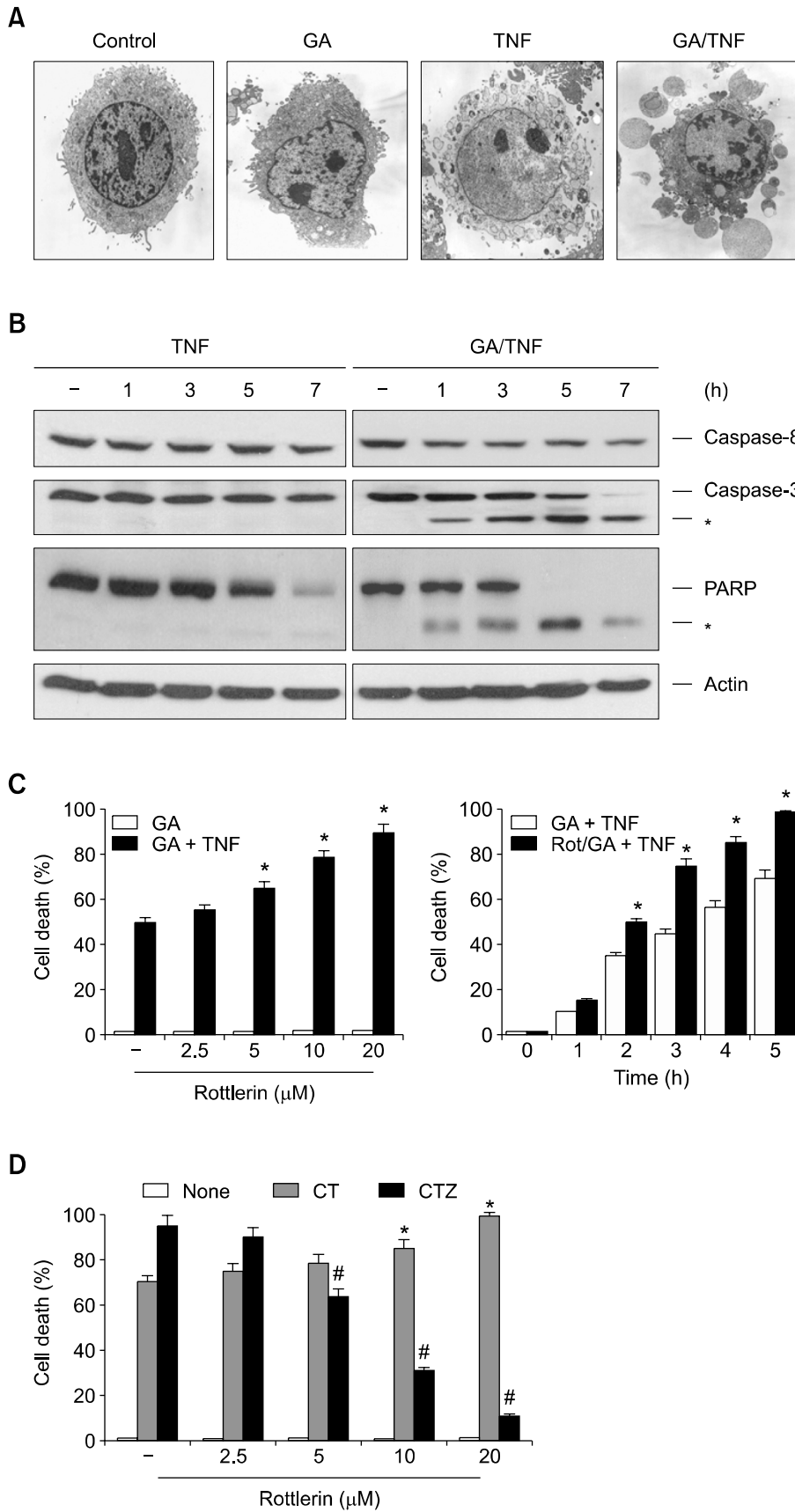


**Figure 2.** Rottlerin sensitizes murine fibrosarcoma L929 cells against GA plus TNF-induced apoptosis. (A) L929 cells were pretreated with GA (1 μM) for 12 h, followed by treatment with TNF (15 ng/ml) for 5 h. Cells were immediately photographed under electron microscopy. (B) L929 cells were pretreated with or without GA (1 μM) for 12 h, followed by treatment with TNF for various times. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against caspase-8, caspase-3, PARP and actin. (C) L929 cells were pretreated with rottlerin at the indicated concentrations (left) and times (right), and further treated with GA (12 h) plus TNF (5 h). Cell death was quantified as described in Figure 1B. *$P <$ 0.05 compared with the GA-plus-TNF-treated group. (D) Mouse embryonic fibroblast (MEF) cells were pretreated with 50 μM z-VAD-FMK for 30 min and then treated with 15 ng/ml TNF or 15 ng/ml TNF plus 10 μg/ml CHX in the absence of presence of rottlerin for various concentrations as indicated. Cell death was quantified as described in Figure 1B. Each bar shows mean ± SE of at least three independent experiments. *$P <$ 0.05, when compared with CHX/TNF-treated group. #$P <$ 0.05, when compared with TNF/CHX/z-VAD-FMK-treated group.

L929 cells. WT-PKCδ and DN-PKCδ expression activated and inhibited PKCδ kinase activity, respectively, as measured by an *in vitro* kinase assay using myelin basic protein as a substrate (Figure 1D, lower panel). However, the overexpression of WT-PKCδ or DN-PKCδ did not influence the protective effect of rottlerin against TNF-induced necrosis (Figure 1D, upper panel). To further rule out the role of PKCδ in modulating sensitivity to TNF-induced necrosis, we investigated whether activating PKCδ with PMA restored the necrotic effect of TNF. However, pre-treating L929 cells with PMA before TNF exposure significantly abrogated necrosis. Moreover, pre-treatment with either bis-indolylmaleimide I or Go6983, both of which are general PKC inhibitors, also did not influence TNF-induced necrosis (Figure 1E). Thus, these results suggest that the anti-necrotic effect of rottlerin upon TNF treatment is not due to a specific PKCδ blockade.

### Rottlerin sensitizes murine fibrosarcoma L929 cells against TNF plus GA-induced apoptotic cell death

The above results clearly show the prevention of TNF-induced necrosis by rottlerin in L929 cells, even though several reports have demonstrated that rottlerin induces apoptosis (Tillman *et al.*, 2003; Kurosu *et al.*, 2007). This suggests that rottlerin may have an opposing regulatory role between apoptotic and necrotic modes of cell death upon death-receptor ligation. To address this issue in the same cell lines, we used the TNF plus geldanamycin (GA)-induced apoptotic model, since TNFR1-mediated necrosis can be reverted to apoptosis when cells are pretreated with an Hsp90 inhibitor (Vanden Berghe *et al.*, 2003). As expected, cell death induced by TNF after pretreatment with GA was morphologically distinct from necrosis, and exhibited apoptotic features such as membrane blebbing, shrinkage of the cytoplasm and chromatin condensation (Figure 2A). This GA-induced shift from necrosis to apoptosis after TNF treatment was confirmed by the activation of caspases and the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP), a typical caspase-3 substrate (Figure 2B, right panel). In contrast, none of these typically apoptotic events were detected in TNF-induced necrosis in the absence of GA (Figure 2B, left panel). Therefore, it is a useful model for investigating the pathway of necrosis and apoptosis upon cell death induced by TNF, alone and in combination with GA. Of interest, in contrast to its anti-necrotic effect, pretreatment with rottlerin sensitized the cells to apoptosis induced by TNF plus GA, and this sensitizing effect was

observed 2 h after co-treatment with TNF and GA (Figure 2C). These results indicate that rottlerin has an opposing role between apoptotic and necrotic modes of TNF-induced cell death. To confirm these observations, we further examined the effect of rottlerin on the apoptotic or necrotic cell death by using a model of MEFs, since apoptotic or necrotic cell death in this MEF cells can be efficiently triggered in the absence or presence of Z-VAD-fmk, a membrane permeable pan-caspase inhibitor, followed by treatment of cells with TNF and cycloheximide (CHX) (Lin *et al.*, 2004; Byun *et al.*, 2006). Pre-treating MEF cells with rottlerin sensitized the cells to apoptosis induced by TNF/CHX, whereas it protected the cells against necrotic cell death induced by treatment with TNF/CHX under the caspase-inhibited condition (Figure 2D), indicating that the effect of rottlerin on TNF-induced necrotic cell death is not specific for L929 cells.

### Rottlerin suppresses mitochondrial $O_2^-$ production induced by TNF and subsequently blocks PARP activation

Caspase-independent necrotic cell death has been proposed to produce ROS which suggests mitochondrial involvement (Garg and Aggarwal, 2002). Given that the first oxygen reduction product generated in mitochondria is $O_2^-$, which can be converted to $H_2O_2$ (Hennet *et al.*, 1993), we examined whether rottlerin interfered with TNF-induced mitochondrial $O_2^-$ production by using a superoxide-sensitive mitochondria-targeted hydroethidine analog, MitoSOX Red (Robinson *et al.*, 2006). MitoSOX Red oxidation, which indicated an increase in mitochondrial $O_2^-$ production, was observed immediately after treatment with TNF, and reached a maximum after 30 min (Figure 3A, upper panel). Importantly, the TNF-induced MitoSOX Red oxidation signal was abolished in the presence of rottlerin or BHA (Figure 3A, middle and bottom panel). This indicates that the protective function of rottlerin against TNF-induced necrosis is derived from its ability to inhibit $O_2^-$ production at the mitochondrial level. Previously, we have demonstrated that the ROS-dependent activation of PARP plays a role in TNF-induced necrosis (Byun *et al.*, 2006). Overactivation of PARP after cellular injury consumes large amounts of NAD, which may cause massive ATP depletion in the effort to resynthesize NAD, and thus shift the mode of cell death toward necrosis (Los *et al.*, 2002). Therefore, if rottlerin is able to protect against cell death via the inhibition of mitochondrial $O_2^-$ production, then it should also impair PARP activation. Accordingly, immunoblotting with anti-PAR antibody, specific for poly (ADP-

**A**



**B**
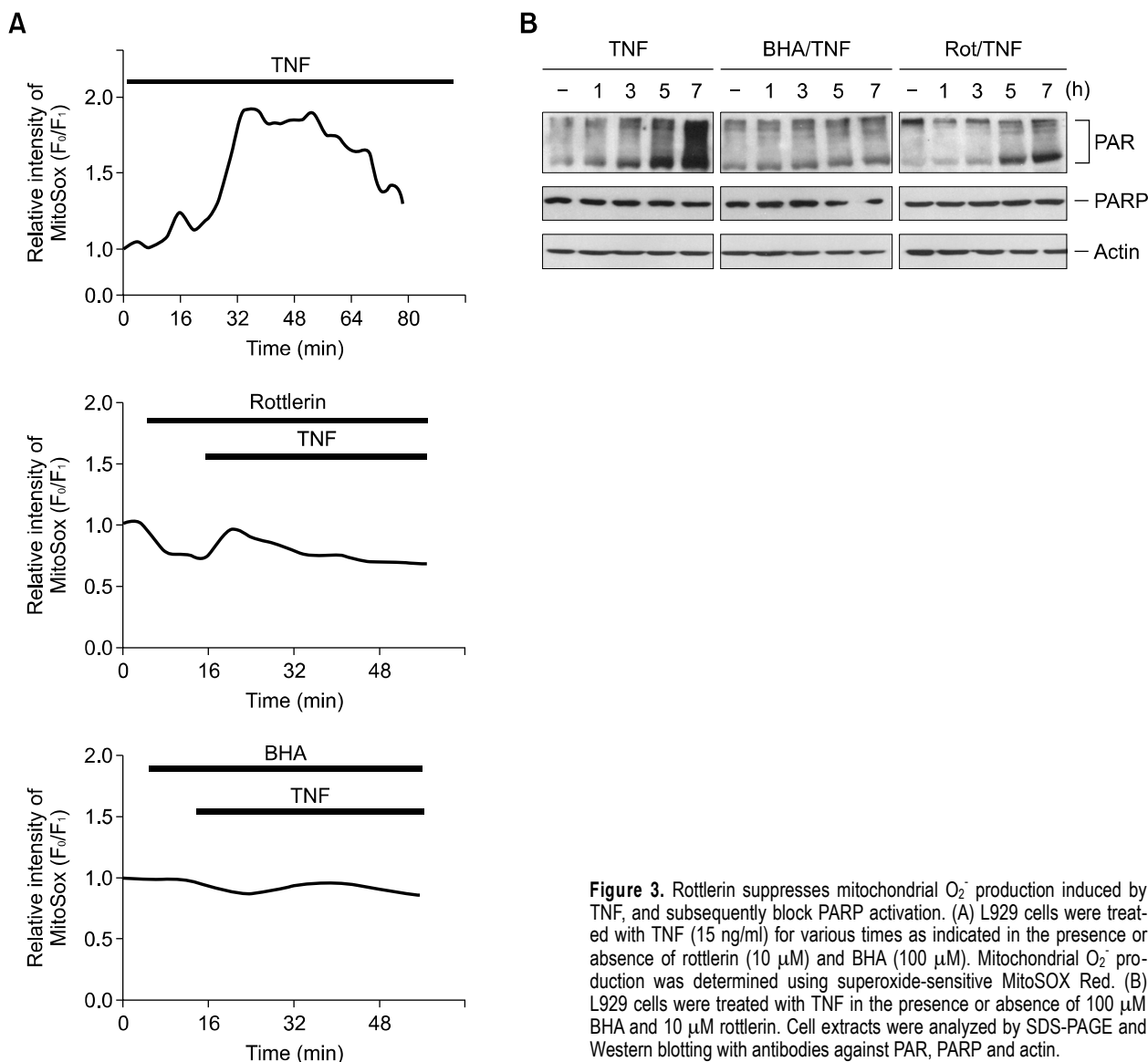


**Figure 3.** Rottlerin suppresses mitochondrial $O_2^-$ production induced by TNF, and subsequently block PARP activation. (A) L929 cells were treated with TNF (15 ng/ml) for various times as indicated in the presence or absence of rottlerin (10 μM) and BHA (100 μM). Mitochondrial $O_2^-$ production was determined using superoxide-sensitive MitoSOX Red. (B) L929 cells were treated with TNF in the presence or absence of 100 μM BHA and 10 μM rottlerin. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR, PARP and actin.

ribose) polymers, showed that treating L929 cells with TNF caused an increase in poly(ADP-ribosyl)-ation after 3 h, after which the level continued to gradually increase (Figure 3B, top panel). Similar to BHA, pre-treatment with rottlerin significantly inhibited these TNF-induced effects, which is consistent with our observation that rottlerin inhibited mitochondrial $O_2^-$ production after TNF treatment.

**Rottlerin suppresses TNF-induced Nox1 activation in murine fibrosarcoma L929 cells**

The data from the above study provide evidence that the protective mechanism of rottlerin against TNF-induced necrosis involves decreased mitochondrial $O_2^-$ production. Another critical issue

remaining to be further investigated is the molecular targeting of rottlerin to inhibit mitochondrial $O_2^-$ production. The Nox family of NADPH oxidase has been implicated as a major source of ROS (Lambeth, 2004; Kim *et al.*, 2005, 2007a). To test whether the ROS generated by NADPH oxidase play a role, we pretreated L929 cells with diphenylene iodonium (DPI), a well-established NADPH oxidase inhibitor in the presence of TNF, and measured mitochondrial $O_2^-$ production. Pretreatment with DPI completely abrogated mitochondrial $O_2^-$ production in TNF-exposed L929 cells (Figure 4A), which suggests that activation of NADPH oxidase by TNF treatment plays a role in mitochondrial $O_2^-$ production. More importantly, a recent study has indicated that Nox1 is the major
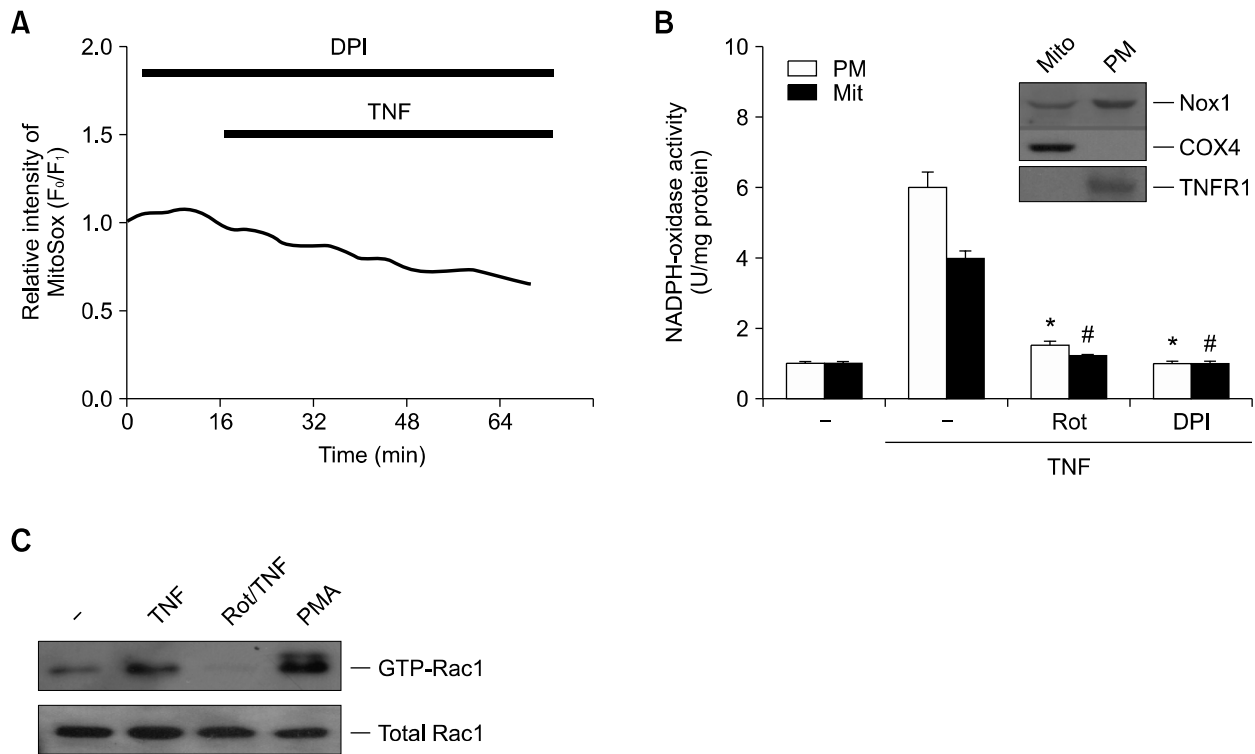
**Figure 4.** Mitochondrial Nox1 NADPH oxidase is expressed in murine fibrosarcoma L929 cells, and rottlerin inhibits Nox1 activity through down-regulation of GTP-bound Rac1. (A) L929 cells were treated with TNF for various times in the presence of 10 μM DPI, and mitochondrial $O_2^-$ production was determined as described for Figure 3A. (B) Mitochondrial and plasma membrane fractions of L929 cells were obtained, and lysates were analyzed by SDS-PAGE, followed by Western blotting with antibodies against Nox1, COX-4 and TNFR1 (upper panel). After treatment with TNF in the presence of rottlerin (10 μM), BHA (100 μM) and DPI (10 μM), NADPH oxidase assays were conducted. (C) L929 cells were transfected with the Xp-tagged Nox1, NOXO1 and NOXA1 expression plasmids, and treated with PMA (50 nM) and/or TNF (15 ng/ml) for 30 min, in the presence or absence of rottlerin (10 μM). The PAK1 pull-down complex was analyzed by SDS-PAGE and Western blotting with antibodies against Rac1.

NADPH oxidase responsible for TNF-induced $O_2^-$ production in non-phagocytic cells (Kim *et al.*, 2007b). Similarly, we were able to detect expression of Nox1, but not Nox2 by Western blotting of L929 cells (data not shown). Thus, Nox1 may be the most likely source of NADPH oxidase activity under conditions of TNF-induced necrosis in L929 cells.

Based on the ability of rottlerin to suppress TNF-induced mitochondrial $O_2^-$ production, it is important to know, for instance, whether Nox1 is expressed in the mitochondria of L929 cells and if so, does rottlerin affect its activity. To address these issues, we used cell fractionation experiments. As expected, the expression of COX-4 unlike that of TNFR1, occurred principally in the mitochondrial fraction, whereas no signal was detected in the plasma membrane fraction (Figure 4B, top panel). In the same lysates, we found that expression of Nox1 occurred in both the mitochondrial and plasma membrane fractions. Furthermore, we also observed that the NADPH oxidase activity in the mitochondrial fraction was increased significantly by TNF treatment, compared to similar activity in the plasma membrane fraction (Figure 4B, bottom panel). This suggests that both mitochondrial and plasma membrane Nox1 are involved in ROS production under necrotic conditions. Notably, treatment with rottlerin, and to a similar extent DPI, which has been shown to suppress mitochondrial $O_2^-$ production (Figure 3A, middle panel), completely inhibited TNF-induced NADPH oxidase activation (Figure 4B, bottom panel). These results suggest that the Nox1 activity correlated well with $O_2^-$ production, as well as necrosis, and that rottlerin inhibits mitochondrial ROS production through Nox1, an important factor driving cells toward necrotic death. Although recent study has demonstrated that Nox1 at the level of receptor signaling complex is involved in TNF-induced necrotic cell death (Kim *et al.*, 2007b), the mitochondrial involvement in this process has not yet excluded. Recent results including ours showed that BHA efficiently suppressed not only mitochondrial $O_2^-$ production (Figure 3A) but also necrosis (Lin *et al.*, 2004;

Byun *et al.*, 2006; Kim *et al.*, 2007b) upon TNF treatment. Therefore, we speculated that the mitochondrial Nox1 is also responsible for ROS production and necrosis by TNF. This conjecture was further supported by the findings of both the potency of DPI to block the mitochondrial $O_2^-$ production, and Nox1 expression in the mitochondrial fraction of L929 cells. However, treatment with rottlerin suppresses the Nox1 activities from both mitochondrial and plasma membrane fractions (Figure 4B), suggesting that rottlerin functions in interfering the Nox1 activities from both sources. Therefore, further studies will be required to elucidate the involvement of exact cellular compartments in Nox1 activation during the necrotic conditions.

Although we have established that the Nox1 is a critical target for anti-necrotic effect of rottlerin in this study, it is still unclear how rottlerin functions to suppress Nox1 activity. Recent reports have indicated that activation of Rac1 by GTP loading functions as a major trigger for activating the Nox1 system (Cheng *et al.*, 2006; Miyano *et al.*, 2006). The Rac1-GTP directly binds to regulatory protein NOXA1 (Nox activator 1) and thus facilitates membrane recruitment of this protein in the presence of NOXO1 (Nox organizer1) (Bánfi *et al.*, 2003; Takeya *et al.*, 2003). Therefore, the GTP-bound form of the small GTPase Rac1 plays a crucial role in Nox1 activation. We next examined the mechanism whereby rottlerin affect the content of endogenous Rac1-GTP, which is required for the formation of active Nox1 complex. Since optimal reconstitution of Nox1 activity requires the co-expression of the NOXO1 and NOXA1 (Ueyama *et al.*, 2006), a pull-down assay was performed using the Rac1-binding domain of protein kinase PAK in Nox1, NOXO1 and NOXA1 coexpressed cells. This binding domain interacts exclusively with the GTP form of Rac1 and CDC42. Consistent with previous data, Rac1 activation assay detected the basal activity of endogenous Rac1 (GTP bound) in unstimulated cells after these were simultaneously transfected with the plasmids of Nox1 and its regulatory subunits, and the treatment of TNF increased the level of activated endogenous Rac1 (Figure 4C). Notably, rottlerin pretreatment failed to induce the GTP-bound form of Rac1 in extracts from TNF-treated cells, despite the fact that comparable amounts of total Rac1 were expressed in each sample. As a control, PMA increased the content of GTP-bound Rac1. Thus these data suggest that rottlerin suppresses the Nox1 activity through affecting the endogenous level of Rac1-GTP in L929 cells. However it is still uncertain whether Rac1-mediated Nox1 activation is oc-

curred in mitochondria and/or plasma membrane. Future studies using isolated components will be able to address this issue properly.

Taken together, our results indicate that ROS generated through activation of Nox1 plays a critical role in TNF-induced necrotic cell death. In addition, rottlerin inhibits Nox1 activity through the down-regulation of GTP-bound Rac1, and subsequent suppression of mitochondrial $O_2^-$ production, therefore inhibiting necrotic cell death after ligation of death receptors such as TNFR.

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