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Minireview

Molecules and Cells

Regulation of Reactive Oxygen Species Generation in Cell Signaling

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Reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide (H_2O_2) are thought to be by-products of aerobic respiration with damaging effects on DNA, protein, and lipid. A growing body of evidence indicates, however, that ROS are involved in the maintenance of redox homeostasis and various cellular signaling pathways. ROS are generated from diverse sources including mitochondrial respiratory chain, enzymatic activation of cytochrome p450, and NADPH oxidases further suggesting involvement in a complex array of cellular processes. This review summarizes the production and function of ROS. In particular, how cytosolic and membrane proteins regulate ROS generation for intracellular redox signaling will be detailed.

INTRODUCTION

Generation of cellular Reactive Oxygen Species (ROS) is induced by both endogenous and exogenous stimuli. Recent evidences convincingly argue that these ROS play an important role in numerous physiological and pathological processes (Droge, 2002; Martindale and Holbrook, 2002). Although high levels of ROS contribute to carcinogenesis and other diseases related to oxidative damage, appropriate levels of ROS have been shown to be indispensible for cell survival, apotosis, and differentiation (Fig. 1). Consistently, various signaling proteins including NF-κB, PI3-K, MAPK, and p53 perform their respective cellular roles in responses to ROS generation (Burdon and Rice-Evans, 1989; Finkel, 1998; Martindale and Holbrook, 2002). Moreover, the relationship between hydrogen peroxide (H_2O_2) and mammalian cell proliferation has been studied in different cell types (Caporossi et al., 2003; Chung et al., 2009; Liu et al., 2002a; Na et al., 2008; Sigaud et al., 2005; Stone and Collins, 2002; Stone and Yang, 2006). For example, reduction of endogenous ROS levels by addition or overexpression of antioxidant proteins inhibit the proliferation of both vascular smooth muscle cells and tumor cells (Caporossi et al., 2003; Liu et al., 2002a; Sigaud et al., 2005; Stone and Collins, 2002). Importantly, inhibition of endogenous ROS generation causes cell-cycle arrest in the G1 phase, demonstrating that ROS steady-state levels are required for entry into the S phase (Chung et al., 2009; Lee et al., 2011; Sekharam et al., 1998). ROS production required for redox signaling is mainly induced by NADPH oxidase, and various growth factors and cytokines stimulate ROS generation by activating this enzyme (Droge, 2002). Although whether ROS originating from the mitochondria contribute to redox signaling is not much known, it is increasingly evident that H_2O_2 released to the cytosol participates in various signaling networks, such as cell-cycle transition and redox balance (Kow-altowski et al., 2009). The objective of this review is to discuss the regulation of ROS production from mitochondria, cytochrome p450, and NADPH oxidase and the implications in cellular signaling.

REGULATION OF MITOCHONDRIAL REACTIVE OXYGEN SPECIES (ROS) GENERATION

Most ROS produced intracellularly originate in the mitochondrial respiratory chain and subsequently produce toxic metabolic byproducts. However, mitochondrial ROS have been increasingly implicated in cellular signaling pathways, including those for survival and cell death. In these processes, many proteins modulate the release of mitochondrial ROS to the cytosol and they are tightly controlled. This ROS release is implicated in the maintenance of redox homeostasis and various cellular signaling pathways. This section summarizes the sources of ROS in the mitochondria and the recent findings regarding how the nuclear encoded proteins regulate ROS release to the cytosol for intracellular redox signaling.

ROS generation in the mitochondrial respiratory chain

One of the main functions of the mitochondria is oxidative ATP production, in which oxygen (O_2) is reduced to water, and consequently the major source of intracellular ROS generation is the mitochondrial respiratory chain (Brand, 2010; Loschen et al., 1973). The respiratory chain consists of five multi-subunits protein complexes located in the mitochondrial IMS (respiratory complexes I-IV and the F_1F_o-ATP synthase) and two factors (cytochrome c, Cyt c; coenzyme Q10) (Galluzzi et al., 2010). During respiration, electrons released from the mitochondrial electron transport chain incompletely reduce O_2 to form superoxide (Chance et al., 1979). Superoxide is converted into H_2O_2 by manganese superoxide dismutase (Mn-SOD) in the mito-

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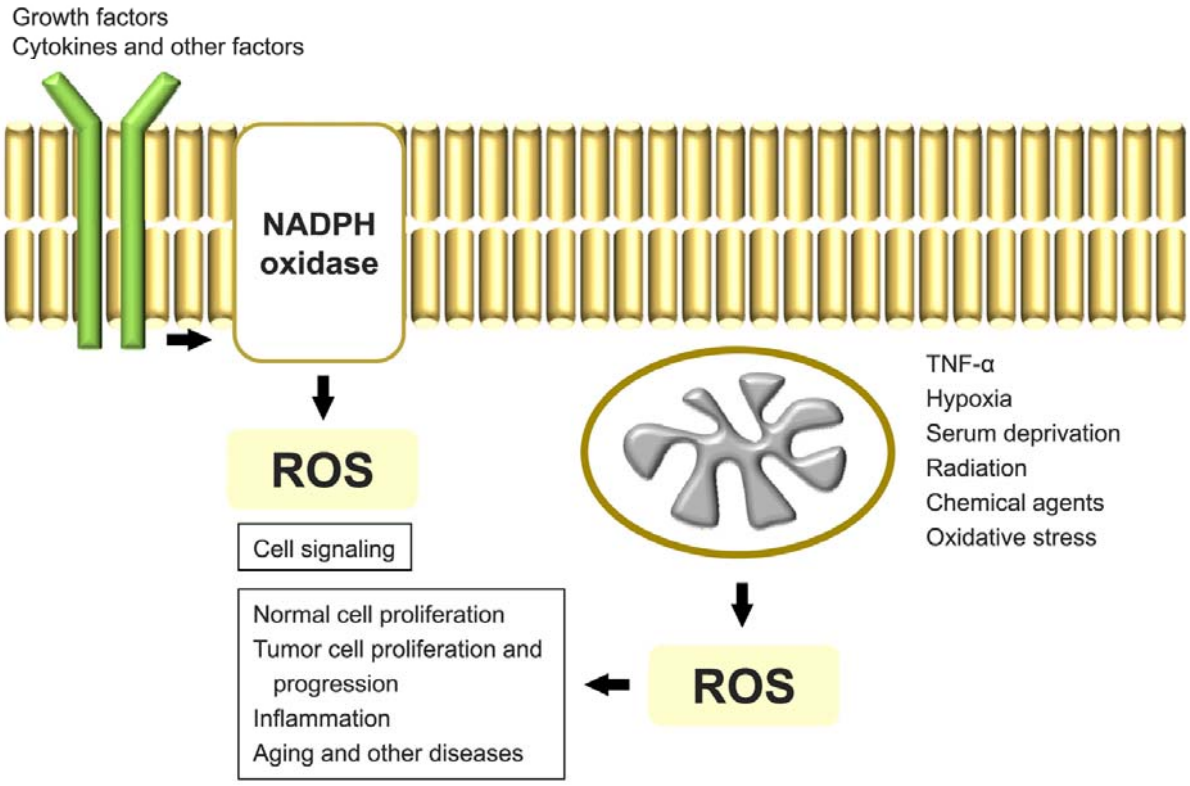

Fig. 1. Mitochondrial ROS accumulation in response to various stimuli. Mitochondria sense various external signals and stresses to induce mitochondrial ROS release to the cytosol. Mitochondrial ROS are indispensible for normal cellular function. However, dysregulation of mitochondrial ROS production or release to the cytosol is implicated in many diseases, especially those involving inflammation.

chondrial matrix or by Cu, Zn-SOD in the IMS of mitochondria (Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001; Weisiger and Fridovich, 1973a; 1973b). The 1-2% of O_2 consumed during respiration is estimated to be incompletely reduced to O_2 to produce superoxide in isolated mitochondria treated with respiratory chain inhibitors (Chance et al., 1979; Kudin et al., 2004). However, 0.12-0.15% of O_2 produces H_2O_2 when palmitoyl-CoA or glutamate/malate are used as substrates (Kudin et al., 2004; Murphy, 2009; St-Pierre et al., 2002). Recently, Brand suggested that mitochondrial superoxide is produced at seven major sites of mitochondria and all sites release it into the matrix (Brand, 2010). Out of the seven sites, complex III (site IIIQo) and glycerol 3-phosphate dehydrogenase also liberate superoxide into the IMS (Chen et al., 2003b; Miwa and Brand, 2005; Muller et al., 2004). Since the two major sites for superoxide production are known to be complexes I and III of the mitochondrial respiratory chain (Chen et al., 2003b; Poyton et al., 2009), these two sites are discussed in this review.

Complex III

Complex III (ubiquinol-cytochrome c oxidoreductase) consists of 11 polypeptides, three hemes and a Fe-S center (Iwata et al., 1998). This complex receives electrons from coenzyme Q (ubiquinol, QH_2) and transfers them to Cyt c. Mitochondrial superoxide is generated through the Q-cycle, as documented in recent papers (Andreyev et al., 2005; Hamanaka and Chandel, 2010; Turrens et al., 1985). Coenzyme Q (ubiquinone, Q) accepts two electrons transferred from mitochondrial complexes I or II to form reduced QH_2. Complex III binds to QH_2 in the intermembrane space-proximal Qo site and transfers these electrons into Cyt c. Because Cyt c can only accept one electron, the first electron is transferred into Cyt c_1 through the Rieske iron-sulfur protein (RISP), resulting in the transient formation of ubisemiquinone ($QH^{\cdot}$). This electron reduces Cyt c and is transferred to complex IV. The other electron of $QH^{\cdot}$ is immediately transferred to the cytochrome b_H center through the cytochrome b_L center of complex III. The oxidized coenzyme Q or $QH^{\cdot}$ can be reduced by cytochrome b_H center at the matrix-proximal Qi site and the resulting QH_2 then transfers the electron into Cyt c_1 in the intermembrane space-proximal Qo site. The superoxide is mainly generated by reaction electron of $QH^{\cdot}$ with O_2 at the Qo site of complex III, liberating it into the IMS (St-Pierre et al., 2002). Antimycin increases superoxide production by blocking electron transfer from the cytochrome b center to Q at the Qi site (Chen et al., 2003b). In contrast, myxothiazol and stigmatellin inhibit superoxide production at the Qo site of complex III (Ksenzenko et al., 1983; Turrens et al., 1985).

Complex I

Complex I (NADH-ubiquinone oxidoreductase) transfers electrons received from NADH to complex III through Coenzyme Q. The mitochondrial superoxide produced by complex I is released into the matrix (St-Pierre et al., 2002). The superoxide at complex I is produced by two mechanisms: NADH-linked forward electron transport and succinate-linked reverse electron transport (Hansford et al., 1997; Kudin et al., 2004; Kushnareva et al., 2002; Kwong and Sohal, 1998; Lambert and Brand, 2004; Liu et al., 2002b; Votyakova and Reynolds, 2001). Higher mitochondrial superoxide generation is observed when the

matrix NADH/NAD+ ratio is high or during reverse electron transport from succinate to NAD+ in isolated mitochondria. However, the superoxide production at complex I under the physiological conditions is controversial. Furthermore, many potential sites of mitochondrial ROS production have been suggested but whether they contribute to mitochondrial ROS production is unclear (Brand, 2010).

Intracellular ROS increase in mitochondria in response to external stimuli

Tumor necrosis factor-α (TNF-α)

TNF-α plays an important role in proliferation, differentiation, and cell death for tissue remodeling during inflammation (Locksley et al., 2001; Wajant et al., 2003). TNF-α signaling is tightly controlled; however, deregulated TNF-α production causes chronic inflammation, which is directly associated with a variety of diseases such as cancer, inflammatory bowel diseases, arthritis, sclerosis, and Alzheimer's disease (Balkwill and Coussens, 2004; Chen and Goeddel, 2002). There are two main pathways for cell survival and death in TNF-α signaling. One is the pro-survival/pro-inflammatory pathway through NF-κB and MAPK activation, which is mediated by TNF-α-induced signaling complex I (Micheau and Tschopp, 2003). After activation of the cell surface receptor (TNF-R1) by binding of TNF-α, the silencer of death domain (SODD) dissociates from TNF-R1 complexes, and the TNF receptor-associated protein with death domain (TRADD) interacts with the cytoplasmic domain of the receptor (Hsu et al., 1995). TRADD recruits TNF receptor-associated factor 2 (TRAF2), receptor-interacting protein 1 (RIP1), and cellular inhibitor of apoptosis proteins to form TNF-α-induced signaling complex I (Chen and Goeddel, 2002; Karin and Lin, 2002). TRAF2 then recruits the IKK complex to the TNF-R1 signaling complex to activate the kinases of the IKK complex via RIP1 (Devin et al., 2000). The activated IKKs phosphorylate IκB to induce the proteolytic degradation of IκB, thereby liberating NF-κB and allowing nuclear translocation (Verma et al., 1995). NF-κB transcription factor regulates expression of the pro-survival proteins, cFLIP, Gadd45b, A20, XIAP, and cIAPs (Amanullah et al., 2003; Papa et al., 2004). NF-κB also induces expression of FHC and Mn-SOD to prevent TNF-R-induced cell death by eliminating ROS (Pham et al., 2004; Sakon et al., 2003). Subsequently, TNF-α induced signaling complex II is formed to activate the other TNF-α signaling pathway that is implicated in cell death, in which ROS, a caspase cascade, and the mitochondria function as downstream mediators. After TRADD, TRAF2, and RIP1 dissociate from the receptor, they interact with Fas-associated death domain protein (FADD) and pro-caspase-8 to form TNF-α-induced signaling complex II (Hsu et al., 1996; Rothe et al., 1994).

ROS have been implicated as mediators of cell survival and cell death triggered by TNF-α signaling. ROS play an important role in TNF-induced cell survival by NF-κB activation (Sulciner et al., 1996). TNF-α-induced ROS production has been also reported to play an important role in TNF-α-induced prolonged JNK activation and cell death (De Smaele et al., 2001; Ichijo et al., 1997; Matsuzawa and Ichijo, 2005; Tang et al., 2001). The generation of ROS in response to signals for TNF-α-induced cell death occurs in the mitochondria (Corda et al., 2001; Lo and Cruz, 1995; Locksley et al., 2001; Matsuzawa and Ichijo, 2005; Meier et al., 1989; Shoji et al., 1995). However, ROS generation originating from NADPH oxidase after TNF-α treatment is also implicated in TNF-α-induced cell death (Kim et al., 2007b). Although many reports show that the main source of TNF-α-triggered ROS is the mitochondria, the mitochondrial origin of ROS formation triggered by TNF-α is not clear. Recently, we demonstrated that Romo1 is a key mediator between TNF-α signaling and the mitochondria for ROS production, as it recruits B-cell lymphoma-extra large (Bcl-X_L) to reduce the mitochondrial membrane potential, resulting in ROS production and apoptosis (Kim et al., 2010). More discussion about Romo1 can be found later in this paper.

Hypoxia

Cells often have to respond and adapt to the unfavorable condition of low oxygen levels (hypoxia). The stability of the hypoxia inducible factors (HIFs) is increased in response to hypoxia and can play a central role in the generation of new vasculature to increase the oxygen supply. Although the exact hypoxia-sensing mechanism is controversial, the mitochondrial electron transport chain is suggested to play an important role in oxygen sensing (Galluzzi et al., 2010). In early studies, it was suggested that mitochondrial production of ROS is involved in hypoxia signaling (Chandel et al., 1998; 2000). Mitochondrial production of ROS is increased in response to hypoxia and these ROS act as upstream mediators of hypoxia-induced signaling process. Treatment with some inhibitors of the electron transport chain inhibits stabilization of HIF-1α in response to hypoxia. In later experiments, it was suggested that mitochondrial complex III is responsible for hypoxia-triggered ROS production (Brunelle et al., 2005; Guzy et al., 2005). These experiments were performed by suppressing the expression of one factor of complex III, by using mitochondrial inhibitors and by using antioxidants. Down-regulation of the RISP blocks hypoxia-triggered HIF-1α stabilization. Hypoxic HIF-1α activation was also suppressed in cells lacking Cyt c, and exogenous treatment with H_2O_2 recovered HIF-1α stability (Mansfield et al., 2005). These studies demonstrate that ROS required for hypoxia signaling are released from mitochondria to cytosol and their origin is mitochondrial complex III. Although there have been many studies about hypoxia-induced ROS generation, how mitochondrial complex III senses hypoxia remains to be investigated, as well as the exact mechanism by which hypoxia induces ROS production at complex III.

Serum deprivation

Serum provides cell growth factors, hormones, and various other factors for most *in vitro* cell cultures (van der Valk et al., 2004). Serum withdrawal triggers cell death and is correlated with ROS production in many cell lines (Charles et al., 2005; Greene, 1978; King et al., 2003; Pandey et al., 2003; Satoh et al., 1996; Zhuge and Cederbaum, 2006). Because serum deprivation is one component of ischemia, there have been several reports about ischemia (Bialik et al., 1999). In serum-deprived neuronal cells, extensive cell death is observed and addition of nerve growth factor (NGF) inhibits the cell death (Ferrari et al., 1995; Greene, 1978). Antioxidant also suppresses cell death, though it cannot recover cell proliferation compared to cells grown in complete media (Ferrari et al., 1995). Although there have been many reports demonstrating that serum deprivation triggers increases in ROS, the mechanism by which serum deprivation induces ROS generation and the source inside cells are not fully elucidated. In our recent paper, we showed that serum deprivation-induced ROS generation, which was measured by MitoSOX, a mitochondrial superoxide indicator, was derived from complex III of the mitochondrial respiratory chain (Lee et al., 2010). Serum deprivation-induced ROS production was inhibited by mitochondrial complex III inhibitors (myxothizol and stigmatellin). However, other mitochondrial complex inhibitors (rotenone, malonate and sodium azide) failed to block the

increase in ROS production in serum-starved cells. We also showed that the serum deprivation-triggered increase in ROS was mediated by Romo1 (Lee et al., 2010). However, the exact mechanism by which Romo1 increases ROS levels in the mitochondria of serum-starved cells requires more extensive investigation.

Other stimuli

Various stresses also stimulate mitochondrial ROS production. Oxidative stress-induced ROS production among external stimuli is significantly implicated in neighboring mitochondrial damage and cell death and this phenomenon is called "ROS-induced ROS release (RIRR)" (Zorov et al., 2000; 2006). The oxidative stress causes the collapse of the mitochondrial membrane potential ($\Delta\Psi m$), resulting in an increase in ROS. The amplified ROS can injure neighboring mitochondria in a positive feedback loop (Kim et al., 2006; Zorov et al., 2000; 2006). Other stresses, including phorbol ester (TPA) and irradiation, also stimulate mitochondria ROS production. TPA-induced ROS production is known to be mediated by NADPH oxidase and/or mitochondria and ROS induced by TPA are associated with tumor cell invasion (Frost et al., 1994; Wu, 2006). 5-FU, arsenic trioxide, cisplatin, paclitaxel, bleomycin, and adriamycin have also been reported to trigger ROS generation (Adler et al., 1999; Huang et al., 2000; Hwang et al., 2001; Marcillat et al., 1989; Miyajima et al., 1997; Pelicano et al., 2003; 2004; Tan et al., 1998).

Regulation of mitochondrial ROS release to the cytosol

Mitochondrially generated ROS are released to the cytosol and participate in a variety of intracellular functions including cell proliferation, cell death, cell cycle, and redox homeostasis (Droge, 2002). H_2O_2 produced by mitochondrial superoxide dismutase can migrate into the cytosol across the membrane by simple diffusion. However, cells more efficiently use mitochondrial ROS by function of the proteins encoded by nuclear genes. In this section, the nuclear encoded proteins that regulate the ROS release to the cytosol will be discussed.

p53

The tumor suppressor protein p53 is a key regulator of cell cycle, senescence, or apoptosis. The main action of the p53 protein is as a transcription factor that induces the expression of target genes and causes cell-cycle arrest, DNA repair, or apoptosis in response to cellular genotoxic stresses (Giaccia and Kastan, 1998; Levine, 1997). Although p53 participates in many cellular activities, only the control of intracellular ROS by p53 will be discussed in this review. p53 differentially regulates the intracellular ROS according to the levels and types of stresses. p53 exerts transcription-dependent pro-apoptotic effects by induction of the pro-oxidant genes. Polyak et al. showed that enhanced p53 activity increased the oxidative stress by inducing its target genes, including two ROS-generating enzymes, NQO1 (quinone oxidoreductase, PIG3) and proline oxidase (POX, PIG6), and resulting in apoptosis (Polyak et al., 1997; Rivera and Maxwell, 2005). BAX, PUMA and p66Shc are also downstream targets of the p53 protein and they induce mitochondrial ROS elevation, which stimulates the opening of the mitochondrial permeability transition pore (PTP) to trigger apoptosis (Giorgio et al., 2005; Liu et al., 2005; Sablina et al., 2005; Trinei et al., 2002). A microarray analysis of H_2O_2-treated human cells showed that one-third of the 48 highly H_2O_2-reponsive genes are related to p53-mediated transcriptional effects (Desaint et al., 2004). Interestingly, p53 plays a role in the maintenance of redox homeostasis by controlling the expression of antioxidant genes, such as SESN1 (mammalian sestrin homologue), SESN2, glutathione peroxidase-1 (GPX1), and TP53INP1 (Cano et al., 2009; Sablina et al., 2005; Stambolsky et al., 2006; Tomko et al., 2006). This antioxidant function of p53 is suggested to be one of the tumor-suppressing mechanisms of p53 (Sablina et al., 2005).

The cellular regulation of p53 mainly occurs by p53-mediated transcriptional activity in the nucleus. However, many studies have reported that a fraction of cellular p53 migrates into the outer mitochondrial membrane in response to a variety of stress signals, including DNA damage and hypoxic stress, and promotes transcription-independent p53-mediated cell death (Chipuk et al., 2004; Dumont et al., 2003; Mihara et al., 2003; Pim and Banks, 2004; Sansome et al., 2001; Yoo et al., 2005). This mitochondrial migration seems to be promoted by monoubiquitylation of p53 (Marchenko et al., 2007). In the mitochondria, p53 interacts with the Bcl-2 family members present at the mitochondrial outer membrane, including Bcl-2 and Bcl-X_L, thereby inducing mitochondrial outer membrane permeabilization (MOMP). MOMP is an upstream event of programmed cell death and is controlled by the Bcl-2 family (Green and Kroemer, 2004; Reed, 2006). p53 appears to bind Bcl-2 or Bcl-X_L to release Bax from their inhibitory complexes to trigger MOMP (Chipuk et al., 2004; Mihara et al., 2003). p53 also interacts with Bak, Bax, and PUMA to induce apoptosis (Chipuk et al., 2004; 2005; Leu et al., 2004). Thus, p53 up-regulates or down-regulates the ROS levels in transcription-dependent or -independent mechanisms.

p66Shc

The Shc adaptor protein family encodes the three isoforms of Shc protein (p46Shc, p52Shc, and p66Shc) that transmit mitogenic signals to RAS (Pelicci et al., 1992). p66Shc is serine phosphorylated in response to external stimuli such as ultraviolet light or ROS (Migliaccio et al., 1999). p66Shc is a downstream mediator of p53 for the elevation of intracellular ROS, Cyt c release and apoptosis (Trinei et al., 2002). Cells lacking p66Shc are resistant to apoptosis triggered by stresses, and $p66^{Shc-/-}$ mice show a 30% increase in life span (Migliaccio et al., 1999). In response to external stresses, p66Shc is phosphorylated on serine 36 residue by the stress-activated kinase PKC-beta and translocated into the mitochondrial IMS (Gertz et al., 2008; Nemoto et al., 2006; Pinton et al., 2007). p66Shc then oxidizes reduced cytochrome c to induce H_2O_2 production, resulting in PTP opening that triggers apoptosis (Giorgio et al., 2005).

The Bcl-2 family

The Bcl-2 family contains proteins with the Bcl-2 homology (BH) region and these proteins are classified into anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins, Bcl-2 and Bcl-X_L, include four BH domains (BH1234). In contrast, the pro-apoptotic proteins Bax and Bak have three BH domains (BH123), and other pro-apoptotic proteins Bad and Bid contain only the BH3 domain (Reed, 2006; Rong and Distelhorst, 2008). The anti-apoptotic and pro-apoptotic functions of the Bcl-2 family are well investigated. However, redox control of Bcl-2 at the outer mitochondrial membrane is not well identified. Although the mechanism of Bcl-2 as an antioxidant is not well elucidated, Bcl-2 has been reported to have an antioxidant effect and this effect is correlated with the anti-apoptotic action of Bcl-2 (Ellerby et al., 1996; Hockenbery et al., 1993; Kane et al., 1993). The antioxidant function of Bcl-2 also regulates cell-cycle arrest at G1 (Deng et al., 2003). Another well-known anti-apoptotic protein Bcl-X_L is reported to have antioxidant action in several

papers. It plays a role in mitochondrial homeostasis by stabilizing $\Delta\Psi m$ (Vander Heiden et al., 1997). Bcl-X_L expression prevents the early decrease in $\Delta\Psi m$ and the subsequent ROS production stimulated by TNF-α (Gottlieb et al., 2000). Bcl-X_L also inhibits TNF-α-induced ROS production and cell death mediated by Romo1 (Kim et al., 2010). Bax localized in the cytoplasm translocates into the outer mitochondrial membrane to induce MOMP, causing Cyt c release in stressful conditions (Wolter et al., 1997). During apoptotic cell death, ROS levels are increased and these are blocked in cells lacking Bax (Kirkland and Franklin, 2001; Kirkland et al., 2002). The mechanism of Bax-triggered increase in ROS levels remains to be studied.

Romo1

ROS produced by the mitochondrial electron transport chain are released to cytosol and are indispensible for cell survival and sometimes the cause of many pathological disorders. Although H_2O_2 is membrane permeable and can be transferred into the cytosol, cells regulate ROS release to the cytosol with some proteins encoded by the nucleus. One excellent example of these is the Romo1 protein. Romo1 acts as a key regulator of mitochondrial ROS release to the cytosol, and its exact mechanisms have begun to be uncovered in the past few years. Romo1 was first identified as the gene expressed differentially in the tumor tissue of a patient who was initially sensitive to chemotherapy but became resistant after a recurrence (Chung et al., 2006). In a subsequent experiment, enforced expression of Romo1 through transfection of the Romo1 gene into several cell lines enhanced ROS levels, as measured by DCF-DA, MitoSOX, or Amplex Red (Chung et al., 2006; 2008). Therefore, it was suggested that Romo1 induces mitochondrial ROS production and that Romo1-triggered ROS production originates in mitochondrial complex III (Chung et al., 2008). It is unlikely that Romo1 generates ROS in response to various stimuli, similar to NADPH oxidase and other enzymes such as xanthine oxidase, cyclooxygenases, and lipoxygenases. Romo1 is located in the outer mitochondrial membrane and its molecular weight is approximately 8.9 kDa, with a single transmembrane domain and no homology domain when compared with these other enzymes (Chung et al., 2006; Kim et al., 2010). Therefore, it was suggested that Romo1 is located in the mitochondrial membrane and that Romo1 acts as a modulator to supply ROS from complex III of the mitochondrial respiratory chain into the cytosol (Chung et al., 2006). If so, how does Romo1 modulate mitochondria ROS release to the cytosol? A recent report showed that TNF-α-induced ROS are associated with Romo1 located in the outer mitochondrial membrane, demonstrating the possible mechanism of ROS release to the cytosol by Romo1 (Kim et al., 2010). In response to TNF-α, TNF complex II interacts with the C-terminus of Romo1. Simultaneously, Romo1 binds to Bcl-X_L to reduce $\Delta\Psi m$, resulting in ROS production and apoptotic cell death. Interestingly, the interaction between Romo1 and Bcl-X_L after TNF-α treatment liberates Bax from Bcl-X_L (Fig. 2). Bax was also reported to induce the mitochondrial production of ROS (Kirkland et al., 2002).

What is the physiological role of Romo1? Although the role of Romo1 has not been fully identified, several reports demonstrate possible roles of Romo1 in the cells. The first possibility is that Romo1 maintains the redox homeostasis for cell survival (Na et al., 2008). Romo1 knockdown decreased the basal levels of ROS and inhibited the cell growth of various normal and tumor cells. The steady-state ROS levels generated from the endogenous Romo1 protein are also indispensable for both normal and cancer cell proliferation, because Romo1 knockdown inhibits cell-cycle transition through inhibition of Erk activation and $p27^{Kip1}$ expression (Chung et al., 2009; Na et al., 2008). Therefore, Romo1 may play an important role in redox signaling during normal cell proliferation. The second possibility is that Romo1 is a molecular bridge between TNF-α signaling and the mitochondria for ROS production that triggers TNF-α-mediated apoptosis (Kim et al., 2010). However, further studies are needed to elucidate the precise role of Romo1 in the TNF-α signaling pathway. The third possibility is that Romo1 expression is up-regulated by various stresses, including serum deprivation, 5-FU, and replicative senescence (Chung et al., 2008; Hwang et al., 2007; Lee et al., 2010). It has been reported that serum withdrawal stimulates mitochondrial ROS production (Ferrari et al., 1995; Satoh et al., 1996). This ROS generation in serum-starved cells is mediated by Romo1 expression (Lee et al., 2010). The last possibility is that Romo1 plays an important role in c-Myc turnover (Lee et al., 2011). The c-Myc protein is up-regulated in response to growth-stimulatory signals to trigger the cell-cycle progression. Subsequently, c-Myc stimulates Romo1 expression. ROS derived from Romo1 expression then trigger Skp2-mediated c-Myc degradation in a negative feedback mechanism.

Romo1 expression is up-regulated in most cancer cell lines, suggesting that increased Romo1 expression might confer chronic oxidative stress to tumor cells (Chung et al., 2006; Hwang et al., 2007). There have been some reported experimental data about Romo1's contribution to carcinogenesis. The enhanced Romo1 expression might induce the endogenous production of ROS, which could cause many DNA mutations in tumor cells, activate some oncogenes or suppress some of the tumor suppressor genes. Indeed, enforced Romo1 expression increases intracellular ROS levels and nuclear DNA damage (Chung et al., 2008). This finding demonstrates that ROS produced by Romo1 modulation move into the nucleus. There is still no direct evidence that Romo1 contributes to human carcinogenesis *in vivo*. Therefore, further studies will be needed to elucidate the precise role of Romo1 during carcinogenesis.

Romo1 could be a promising drug target, and drug development targeting Romo1 is possible in TNF-α pathobiology. The balance between survival and death after TNF-α stimulation is well controlled by crosstalk between the pro-survival pathway and the pro-apoptotic pathway. Deregulated activation of TNF-α signaling has been reported to contribute to a variety of diseases (Chen and Goeddel, 2002). So far, many agents that block TNF-α function, for example antibodies specific for TNF-α such as Infliximab, Adalimumab, Golimumab, and CDP571 or antibodies specific for TNF-R2 including Etanercept, have been developed, and the clinical trials were successful (Zidi et al., 2010). Since these agents bind to TNF-α or TNF-R to inhibit the physiological function, they may elicit unwanted outcomes including lymphoma development and infection (Bongartz et al., 2006). Romo1 expression is increased in pathological conditions, such as in senescent cells and cancer cells, and TNF-α-induced ROS production is involved in inflammatory diseases (Chung et al., 2006; 2008; Han et al., 2009). Therefore, we suggest that Romo1 is a novel and promising target for the development of new anti-inflammatory drugs that target TNF-α signaling. These agents targeting Romo1 can block the TNF-α-derived ROS production but not the complex I-mediated pathway of TNF-α signaling. The agents can also be utilized as inflammatory agents that do not remove the already-produced ROS but rather block the origin of ROS production. We expect that a Romo1 antagonist that inhibits the cell death pathway of TNF-α signaling will be more effective than current antioxidants for curing diseases associated with TNF-α.

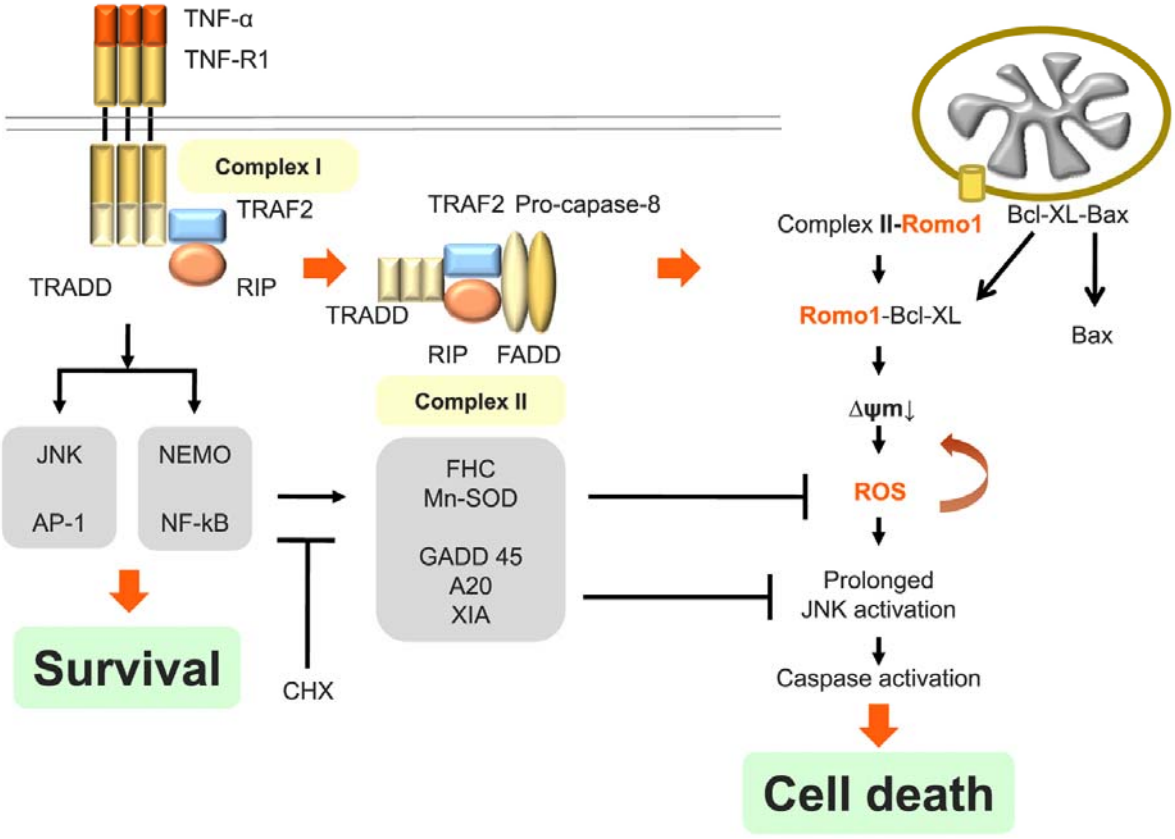

Fig. 2. TNF-α-induced ROS production through Romo1 and Bcl-X_L. In response to TNF-α, TNF-α signaling complex II is translocated into the mitochondria and interacts with the C-terminal region of Romo1. Simultaneously, Romo1 recruits Bcl-X_L and mediates the early TNF-α-induced decrease of ΔΨm, probably by binding to Bcl-X_L and preventing its function. Subsequently, TNF-α-induced decrease of ΔΨm triggers ROS production, which leads to the late decrease of ΔΨm and ROS production. Eventually, the amplified ROS generation induces prolonged JNK activation and apoptotic cell death.

REGULATION OF CYTOCHTOME P450

The cytochrome P450 (CYP) superfamily is a large group of enzymes that catalyze the oxidation of hydrophobic organic molecules. CYP enzymes have been identified in all branches of life (Danielson, 2002). The substrates of mammalian CYP enzymes include metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotic substances such as drugs and other toxic chemicals. The most common reaction catalyzed by mammalian CYPs is a monooxygenase reaction in which CYPs bind oxygen and use one atom of oxygen to hydroxylate their substrate (RH) and the other to produce H_2O:

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$$

The two electrons required for the conversion of oxygen atom to H_2O are generally furnished by NADPH. Two types of routes are known for the passage of the electrons from NADPH to mammalian CYPs, which are present in both endoplasmic reticulum (microsomes) and mitochondria. In the monooxygenation reaction catalyzed by microsomal CYP, electrons are passed to CYP via cytochrome p450 reductase which is comprised of two coenzymes, FAD and FMN (Degtyarenko and Kulikova, 2001). In contrast, the electron transfer to mitochondrial CYPs depends on adrenodoxin reductase, which is an FAD-containing flavoprotein and adrenodoxin, which is a ferredoxin type iron-sulfur protein (Ziegler et al., 1999).

CYP enzymes share a common structural topology with a heme group in their active site and derive their name from the absorption maximum of their reduced carbon monoxide complex at 450 nm (Coon, 2005).The heme iron is tethered to the CYP protein via a thiolate ligand derived from a cysteine residue. This cysteine and several flanking residues are highly conserved in CYPs. In the substrate-free form, CYPs have their heme iron predominantly in its six-coordinated low spin state (Fig. 3). The substrate (RH) binds to the active site of the enzyme, in close proximity to the heme group, on the side opposite to the cysteine. The bound substrate induces a change in the conformation of the active site, displacing a water molecule from the heme iron, shifting the spin equilibrium towards to the five-coordinated high spin form (step 1). The change in the spin state favors the transfer of an electron from NADPH *via* cytochrome P450 reductase or adrenodoxin, reducing the ferric heme iron to the ferrous state (step 2). The ferrous CYP then binds an oxygen molecule to form an oxy-complex of CYP (step 3). A second electron is transferred via either cytochrome P450 reductase or adrenodoxin to the oxy-complex of CYP to give a peroxy-complex (step 4). The peroxo group formed in step 4 is rapidly protonated twice and results in the heterolytic cleavage of the O-O bond, producing one water molecule, and forming a highly reactive species commonly referred to as Compound I (Rittle and Green, 2010) (Step 5). The activated

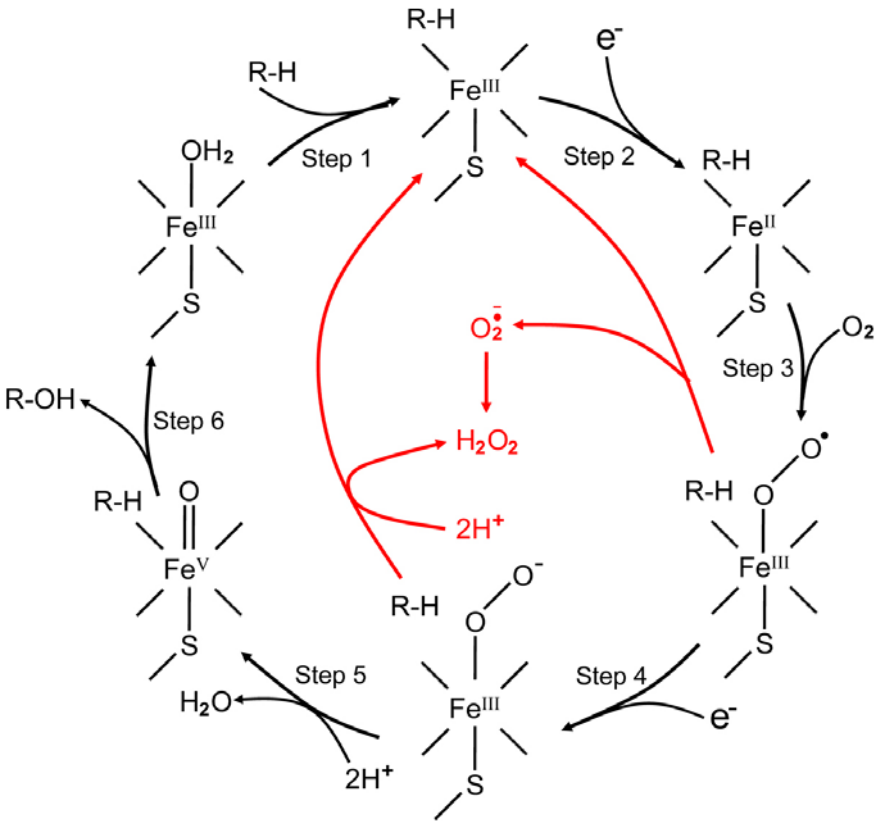

Fig. 3. The catalytic cycle of cytochrome p450 (CYP) with the main cycle that leads to substrate hydroxylation (shown in black arrows) and with the leaky branches that lead to ROS production (shown in red arrows). See text for details.

oxygen atom of compound I is then inserted into the substrate molecule to produce ROH (step 6).

During the catalytic cycle, a significant portion of the activated oxygen is released from the catalytic intermediate to produce superoxide or H_2O_2 (Gorsky et al., 1984; Hanukoglu, 2006; Zangar et al., 2004). One such intermediate is the one-electron reduced oxy-complex (produced in step 3), which decays to produce superoxide (branch 1). The other intermediate is the two-electron-reduced peroxy-complex, which is protonated with the formation of H_2O_2 (branch 2). Continuous production of such ROS is an inevitable result of NADPH consumption by CYP both in the presence and in the absence of substrates. However, the partition of these branches depends on many conditions, such as the types of CYP and substrate, pH, ionic strength, oxygen concentration, and other factors (Gorsky et al., 1984; Hanukoglu, 2006; Zangar et al., 2004). The efficiency of electron transfer from NADPH through the electron carriers to the CYP for monooxygenation of substrate is called the degree of coupling. If a portion of electrons are transferred to other acceptors, such as O_2 leading to production of ROS, then the process is referred to as "leaky" electron transfer. Although the degree of coupling (or leakiness) varies for different CYP species, it is usually less than 50% (Gorsky et al., 1984; Hanukoglu, 2006; Zangar et al., 2004; Zhukov and Archakov, 1982). Due to such leakiness, the rate of NADPH and oxygen consumption by CYPs is only weakly dependent on the presence of substrates. Even in the absence of any substrates, the CYP electron-transfer chain continues to oxidize NADPH and produce ROS.

The microsomal CPYs and their reductase are bound to the outer surface of the ER membrane by transmembrane anchors, whereas the components of mitochondrial CYP system (CYP, adrenodoxin, and adrenodoxin reductase) are located on the matrix side of the inner membrane mitochondria membrane (Gorsky et al., 1984; Hanukoglu, 2006; Hanukoglu et al., 1990; Zangar et al., 2004). The amount of microsomal CPY system components and the intensity of electron transfer processes in the ER are often underestimated. Despite the prevailing impression that most electron transfer processes in the cell are localized in the mitochondria, more than 60% of the electron-transfer hemoproteins and about 20-30% of membrane-bound flavoproteins are localized in the ER of the liver cell (Venditti et al., 1998). Although the amount of CYPs in other tissues is usually lower than in liver (McKinnon and McManus, 1996), it commonly remains comparable with the content of mitochondrial electron carriers. In steroidogenic cells, the amounts of mitochondrial CYP system components are as much as 10 times higher than other electron-transfer chain proteins (Hanukoglu, 2006). Thus, even if the CYP systems leak electrons at low rates, their total capacity for ROS generation could be high, especially in the liver and steroidogenic tissues.

ROS produced by CYP systems can potentially cause lipid peroxidation, cell toxicity, and death. Mitochondria are known to have a rich repertoire of antioxidant enzymes such as manganes superoxide dismutase (Mn-SOD), and glutathione peroxodase 1 and 5, as well as small antioxidant molecules such as ascorbic acid, tocopherol, and carotene to prevent the toxic effect of ROS (Hanukoglu, 2006). Peroxiredoxins (Prxs) are peroxidases that catalyze the reduction of reactive oxygen species (ROS) (Rhee and Woo, 2011). There are six different mammalian isoforms Prx I to VI), each with different subcellular localization**.** Prx III is found exclusively in mitochondria and plays an essential role in mitochondrial homeostasis (Chang et al., 2004). The active site cysteine residue of members of the 2-Cys Prx subgroup (Prx I to IV) of Prxs is hyperoxidized to cys-

teine sulfinic acid (Cys-SO_2) during catalysis with concomitant loss of peroxidase activity. Reactivation of the hyperoxidized Prx is catalyzed by sulfiredoxin (Srx) (Rhee and Woo, 2011).

It is well known that ethanol consumption induces the accumulation of cytochrome P450 2E1 (CYP2E1), a major contributor to ethanol-induced ROS production in the liver (Gong and Cederbaum, 2006). It was shown recently that chronic ethanol feeding markedly increased the expression of Srx in the liver of mice in a largely Nrf2-dependent manner (Bae et al., 2010). Among Prx I to IV, only Prx I was found to be hyperoxidized in the liver of ethanol-fed wild-type mice because a large fraction of Prx I is located together with CYP2E1 at the cytosolic side of the ER membrane (Bae et al., 2010). The selective role of Prx I in ROS removal is thus likely attributable to the proximity of Prx I and CYP2E1. The pivotal functions of Srx and Prx I in protection of the liver in ethanol-fed mice were evident from the severe oxidative damage observed in mice lacking either Srx or Prx I (Bae et al., 2010).

REGULATION OF NADPH OXIDASE

General structure of NADPH oxidase

Growing evidences indicate that transient ROS can be made by receptor-mediated enzymatic processes, but not simple electron-leaking from mitochondrial membrane. Agonist-mediated ROS generation for host defense has been extensively studied in $gp91^{phox}$/NADPH oxidase 2 (Nox2) of phagocytic cell. However, after identification of homologs of $gp91^{phox}$ (Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2) in non-phagocytic cells the function of generated ROS has been extended into understanding of various cellular events including cell growth, differentiation, apoptosis, and immune responses. Since $gp91^{phox}$ (renamed as Nox2) was identified in phagocytic cells, six homologues of Nox2 in non-phagocytic cells to date have been reported. Seven Nox isozymes are all single polypeptides and can be divided into three types based on their functional domains. The catalytic protein Nox2 serves as the core protein of NADPH oxidase complex in phagocytic cells (Segal, 2005). The carboxyl terminal region of Nox2 contains NADPH- and FAD-binding sites homologues to ferredoxin-$NADP^+$ reductase (FNR) (Segal et al., 1992; Sumimoto et al., 1992) and NH_2-terminal region of Nox2 bears six transmembrane α-helical domains containing tandem heme groups homologues to ferric reductase (F_{re}) (Finegold et al., 1996) (Fig. 4). Active form of Nox2 mediates an electron transport from NADPH (K_m for NADPH is 40 μM; K_m for NADH is 2.5 mM) through FAD and two hemes to oxygen finally to generate superoxide anion (Clark et al., 1987). Nox1, Nox3, and Nox4 have similar structure with Nox2 and then categorized into prototype. Nox5 and Duox1/2 contain EF hand structure and appear to be activated by intracellular calcium (Figs. 4D and 4E). Oxidase domain of Nox5 constitutes prototype structure and additional NH_2-terminal region bearing four EF-hands (three canonical motifs and one uncanonical motif) as regulatory domain. Increasing intracellular calcium binding to EF-hands in Nox5 induces conformational change leading to intramolecular interaction of EF-hands with oxidase domain in NH_2-terminal region (Banfi et al., 2004). Because calcium affinity in EF-hands of Nox5 is relatively low (EC_{50} of calcium is 1 μM), additional regulatory mechanism is required for calcium sensitivity. Either phosphorylation of FAD-binding domain or association of calmodulin with NADPH-binding site in Nox5 increases calcium sensitivity (Jagnandan et al., 2007; Pandey et al., 2011; Tirone and Cox, 2007). Duox isozymes contain two EF-hands in NH_2-terminal region (De Deken et al., 2000). Intracellular calcium binds to EF-hands and induces conformational changes resulting in Duox activation. Partial digestion of EF-hands by protease treatment shows increased Duox activity suggesting that EF-hands of Duox act as negative regulator (Dupuy et al., 1992). Peroxidase-like domain of Duox is located in extracellular NH_2-terminal region. Although peroxidase activity generally requires for heme group, peroxidase-like domain of Duox does not contain to heme group. Because critical histidine residues in conserved region were replaced by serine in peroxidase-like ectodomain of Duox, it appears to be inactive form (Meitzler and Ortiz de Montellano, 2009). It has been well established that Nox enzyme catalyzes NADPH-dependent one electron reduction of oxygen to generate superoxide anion (O_2^-) that could be dismutated by spontaneously or enzymatic reaction to generate H_2O_2 (Nakamura et al., 1989; 1991). However, Duox in thyroid and lung airway cells appears to be source of H_2O_2 (Dupuy et al., 1991; Forteza et al., 2005; Leseney et al., 1999). EPR (Electron paramagnetic resonance) experiment with spin trap BMPO demonstrated that Duox generates H_2O_2 directly through unknown mechanism (Ameziane-El-Hassani et al., 2005).

Regulation of $gp91^{phox}$/Nox2

The catalytic protein $gp91^{phox}$ (renamed as Nox2) serves as the core protein of NADPH oxidase complex in phagocytic cells (Segal, 2005). Catalytic core protein, $gp91^{phox}$/Nox2 needs to form a complex with one membrane-integrated protein, $p22^{phox}$, three cytosolic proteins- $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$- and small G-protein Rac. Integral protein $p22^{phox}$ tightly associates with catalytic protein Nox2 to form cytochrome b_{558} (Borregaard et al., 1983; Huang et al., 1995) providing for the stabilization of Nox2 and recruitment of cytosolic protein $p47^{phox}$. Expression of Nox2 is undetectable in phagocytes from chronic granulomatous disease patients with defect of $p22^{phox}$ suggesting heterodimerization of Nox2 with $p22^{phox}$ is critical for the stabilization of Nox2 expression (Dinauer et al., 1990; Parkos et al., 1989; Stasia et al., 2002) (Fig. 4A). Moreover, $p22^{phox}$ protein seems to be important integral partner for other Nox isozymes such as Nox1, Nox3, and Nox4 (Ambasta et al., 2004; Kawahara et al., 2005; Martyn et al., 2006; Takeya et al., 2003; Ueno et al., 2005). The $p22^{phox}$ protein contains proline-rich region (PRR) in carboxyl terminal region providing an interacting site for tandem Src homology 3 (SH3) domains of $p47^{phox}$ (DeLeo et al., 1995; Leto et al., 1994).

The $p47^{phox}$ protein acts as adaptor molecule for the formation of NADPH oxidase complex. It contains phagocyte oxidase (PX) domain in NH_2-terminal region for targeting phosphoinositide lipids in membrane, tandem SH3 domains in central region for binding with $p22^{phox}$, autoinhibitory region (AIR) next by SH3 domain, and proline-rich region (PRR) in carboxyl terminal region for recruiting SH3 domain of $p67^{phox}$. In resting stage, tandem SH3 domains of $p47^{phox}$ protein form intra-molecular interaction with AIR interfering interaction with other proteins (Groemping et al., 2003; Yuzawa et al., 2004a; 2004b). Upon response to stimuli, extensive phosphorylation of AIR by various protein kinases (PKC, Erk, p38MAPK, Pak, and Akt) (Chen et al., 2003a; El Benna et al., 1994; 1996; Fontayne et al., 2002; Martyn et al., 2005) leads to conformational change in $p47^{phox}$ which renders interaction of tandem SH3 domain of $p47^{phox}$ with $p22^{phox}$ and PRR in carboxyl terminal region to SH3 domain of $p67^{phox}$ protein (Ago et al., 2003). Conformational change by phosphorylation also allows PX domain accessible to phospho inositide lipid such as phosphtidylinositol (3,4)-bisphosphate [$PI(3,4)P_2$], a product of phosphatidylinositol 3-kinase (PI3K), in membrane (Ago et al., 2003). PX domain also has a binding activity with acidic phospholipids including phosphatidic acid

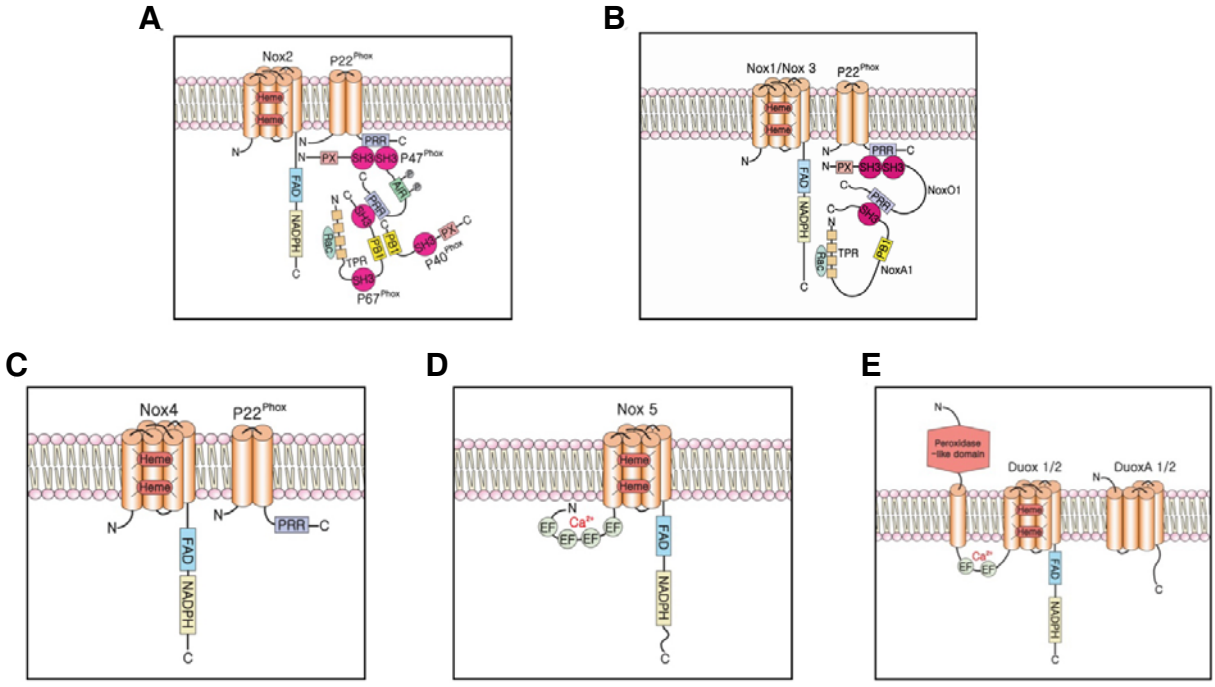

Fig. 4. Structure and activation of NADPH oxidase (Nox) isozymes. Nox isozymes contain six transmembrane α-helical domains containing tandem heme binding sites in NH_2-terminal region and NADPH- and FAD-binding sites in long carboxyl terminal region. Nox1, Nox3, and Nox4 have similar structure with Nox2 and then categorized into prototype. Nox5 and Duox1/2 bear EF hand structure in NH_2-terminal region. Additional peroxidase-like domain of Duox is located in extracellular NH_2-terminal region. $p22^{phox}$ protein contains proline rich region (PRR) providing a binding site for $p47^{phox}$ and NoxO1. See text for details.

(PA) and phosphatidylserine (PS) which support membrane binding of $p47^{phox}$ (Karathanassis et al., 2002; Stahelin et al., 2003).

Small G-protein, Rac protein is a essential molecule for the action of Nox2 (Abo et al., 1991; Knaus et al., 1991; Mizuno et al., 1992). The $p67^{phox}$ protein contains four tetratricopeptide repeats (TPR) for a binding site of G-protein Rac (Koga et al., 1999; Lapouge et al., 2000). TPR domains consist of 34 amino acid repeat (Lamb et al., 1995; Ponting, 1996) and two hairpin loop connecting TPR1-TPR2 and TPR2-TPR3 provide an interacting site for GTP-bound form of Rac (Lapouge et al., 2000). Truncated domain of $p67^{phox}$ (amino acids 1-199) is completely loss of Nox2 activation, whereas longer stretch domain of $p67^{phox}$ (amino acids 1-212) restores their activity (Hata et al., 1997). Short stretch region (amino acids 201-210) of $p67^{phox}$ was named as activation domain (AD) (Han et al., 1998). Moreover, mutant V204A of $p67^{phox}$ shows null function of Nox2 activation (Han et al., 1998; Price et al., 2002). Thus, TPR domain in NH_2-terminal region and short stretch of activation domain are essential for $p67^{phox}$ function. Two SH3 domains are located into carboxyl terminal region. Although the function of NH_2-terminal SH3 is unclear, carboxyl terminal SH3 domain can specifically interact with PRR of $p47^{phox}$ leading to translocation of Nox2 protein complex (de Mendez et al., 1997; Hata et al., 1997). The $p67^{phox}$ protein contains PB1 domain in between two SH3 domains mediating molecular interaction with PB1 domain of $p40^{phox}$ (Kuribayashi et al., 2002). In crystal structure of $p67^{phox}$ and $p40^{phox}$, interaction of Lys355 of $p67^{phox}$ with Asp289, Glu291, and Asp293 of $p40^{phox}$ contributes intermolecular association (Wilson et al., 2003). Translocation of $p40^{phox}$ is mediated by PX domain located in NH_2-terminal region (Ueyama et al., 2007). PX domain in $p40^{phox}$ can specifically bind to phosphatidylinositol 3-phosphate [PI(3)P], highly enriched in the phagosomal membrane (Bravo et al., 2001; Kanai et al., 2001; Stahelin et al., 2003). However, the function of SH3 domain in $p40^{phox}$ remains to be elucidated.

Regulation of Nox1

Nox1 is firstly identified as a homologue of $gp91^{phox}$/Nox2 from non-phagocytic cells, colon epithelium (Suh et al., 1999) and requires for one integral protein, $p22^{phox}$ and two cytosolic proteins, Nox organizer 1 (NoxO1) as homologue of $p47^{phox}$, Nox activator 1 (NoxA1) as homologue of $p67^{phox}$, and small G-protein Rac (Cheng et al., 2006; Geiszt et al., 2003a; Kawahara et al., 2005). Function of $p22^{phox}$ for Nox1 complex is similar to that for Nox2 (Fig. 4B). The $p22^{phox}$ protein interacts with catalytic core protein Nox1 and recruits NoxO1 protein through the interaction of PRR of $p22^{phox}$ with SH3 domain of NoxO1 (Takeya et al., 2003). NoxO1 protein acts as central scaffolding molecule in Nox1 activation. Structure of NoxO1 is very similar with that of $p47^{phox}$ protein except lacking of AIR domain. It contains PX domain in NH_2-terminal region for binding of phosphoinositide lipid, tandem SH3 domains for PRR of $p22^{phox}$, and PRR in carboxyl terminal region for the interaction with SH3 domain of NoxA1. Because NoxO1 protein lacks of AIR, free tandem SH3 domains of NoxO1 interact with PRR of $p22^{phox}$ providing translocation of NoxO1 to membrane (Cheng and Lambeth, 2004). Therefore, Nox1 appears to be constitutively active. NoxA1 protein contains four TPR domains in NH_2-terminal region for Rac binding sites and one SH3 domain located in central region for interaction with PRR of NoxO1 as similar as $p67^{phox}$ (Cheng et al., 2006; Takeya et al., 2003). However, there is no supporting evidence that PB1 domain of NoxA1 can interact with $p40^{phox}$ or its homologue.

The multiprotein complex formation seems to be necessary not only for the activation but also for the regulation of Nox activity. The c-Src substrate protein Tks4 (tyrosine kinase substrate with four SH3 domains) is functional member of a $p47^{phox}$-related organizer superfamily and interact with PRR of NoxA1 protein through SH3 domain-mediated interaction resulting in Nox1 activation (Gianni et al., 2011). Interestingly, Src-mediated phosphorylation of Tyr110 on NoxA1 and of Tyr508 on Tks4 stimulate their association and induce Nox1-dependent ROS generation (Gianni et al., 2010). However, Tks proteins do not support Nox2 and Nox4 activities. It has been shown that PKA phosphorylates Ser172 and Ser461 of NoxA1 providing a binding site for 14-3-3 protein (Kim et al., 2007a). Association of phosphorylated NoxA1 with 14-3-3 results in dissociation of NoxA1 from Nox1 protein complex and cessation of superoxide anion production. In addition, recent two reports showed that the action of Erk leads to phosphorylation of Ser282 of NoxA1 and that phosphorylation of Ser282 in turn results in negative regulation of Nox1 activity (Kroviarski et al., 2010; Oh et al., 2010).

Regulation of Nox3 and Nox4

Since Nox3 gene was identified from fetal tissues, substantial evidence indicated that the Nox3 protein is expressed in inner ear. Positional cloning experiment demonstrated that defect of Nox3 results in head tilt mice (Nox3*het*) through impairment of otoconia formation in inner ear (Paffenholz et al., 2004). After head-tilt mice were due to non-function of Nox3 in inner ear, these mice models (nmf333 mice with defect of $p22^{phox}$, *hstl* mice with defect of NoxO1) allow understanding the regulatory mechanism in which $p22^{phox}$ and NoxO1 are required for activation of Nox3 (Kiss et al., 2006; Nakano et al., 2008) (Fig. 4B). Although the transfection with $p67^{phox}$, $p47^{phox}$, and Rac1 protein stimulates Nox3 (Cheng et al., 2004; Miyano et al., 2009; Ueyama et al., 2006), the regulatory mechanism of Nox3 remains to be elucidated.

Nox4 protein is typical catalytic subunit which requires for only $p22^{phox}$ and seems to be constitutively active (Ambasta et al., 2004; Martyn et al., 2006) (Fig. 4C). Substantial evidence indicated that the expression of Nox4 is inducible and the activity is dependent on its expression level. Although molecular partner for Nox4 to date has not been reported, polymerase delta-interacting protein (Poldip2) as a $p22^{phox}$-interacting protein stimulates Nox4 activity in SMC (Lyle et al., 2009). However, the molecular mechanism and function of Nox4 by Poldip2 remains to be determined.

Regulation of Nox5 and Duox1/2

No binding protein for Nox5 has been reported. Integral protein $p22^{phox}$ can not be interacted with Nox5 and knockdown of $p22^{phox}$ does not affect Nox5 activation (Brar et al., 2003; Kawahara et al., 2005). Activation of Nox5 protein only requires for intracellular calcium because it contains four EF-hands in NH_2-terminal region (Banfi et al., 2001; 2004) (Fig. 4D). Moreover, PMA-dependent phosphorylation of Nox5 on Thr494 and Ser498 allows increasing calcium sensitivity (Jagnandan et al., 2007). It is likely that concerted action of phosphorylation and calcium binding is involved in the regulation of Nox5 activity. However, studies of molecular function of Nox5 are limited because Nox5 does not express in mouse.

As like as Nox5, Duox isozymes requires intracellular calcium mobilization for their activation through calcium binding to EF-hands (Ameziane-El-Hassani et al., 2005). The association induces conformational changes resulting in Duox activation. Recently, Duox activators, DuoxA1 and DuoxA2, identified as maturation factor are essential for the delivery of Duox from endoplasmic recticulum to plasma membrane (Grasberger and Refetoff, 2006) (Fig. 4E). DuoxA1 and DuoxA2 are located on chromosome 15 in a head to head arrangement with Duox1 and Duox2, respectively, suggesting that the expression of Duox1/DuoxA1 and Duox2/DuoxA2 are coordinated by a common transcription (Pachucki et al., 2004). DuoxA provides Duox translocation from ER-Golgi to plasma membrane as well as stimulates glycosylation of Duox protein during subcellular translocation of Duox providing stable conformation between Duox and DuoxA (Grasberger and Refetoff, 2006). Moreover, Leto and his colleagues showed that functional pairing of Duox1/DuoxA1 and Duox2/DuoxA2 contributes H_2O_2 production in reconstitution experiments (Morand et al., 2009).

Function of Nox in host defense

The first described microbicidal function of Nox was in phagocytic cells such as macrophages and neutrophils highly expressing Nox2. Superoxide anion generated by NOX isozymes is not much contributed in microbial killing because it is less reactive. Instead, phagocytic cells utilize more relevant mode in which HOCl is generated in sufficient concentrations to kill bacteria by combined action of H_2O_2 and secrected myeloperoxidase (MPO) in phagosome (Jiang et al., 1997). However, genetic deficiency in MPO does not able to show antimicrobial defect as like as shown in the defect of $gp91^{phox}$ at an increased risk of infection (Kutter et al., 2000). While MPO has a contribution to Nox-mediated antimicrobial mechanisms, several lines of evidence suggest that there is MPO-independent microbicidal pathway. In addition, Nox2 activation leads to rise in phagosomal pH through the generation of hydroxyl ion from superoxide anion, leading to a charge build-up that requires compensation (Henderson et al., 1987; Schrenzel et al., 1998). Most of the charge compensation not only occurs through H^+ channels opening but also K^+ influx (Ahluwalia et al., 2004; Reeves et al., 2002; Segal, 2005). Moreover, K^+ influx could contribute to bacterial killing through changes in phagosomal osmolarity and activation of cationic proteases such as elastase and cathepsin G (Reeves et al., 2002). The host defense concept in macrophage suggested that both of HOCl generated by concerted action of Nox2 with MPO and the charge compensation by K^+ influx are essential for microbial killing. Based on this mechanism, it is clear that chronic granumatous disease (CGD) is caused by the genetic mutations of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ and fails to generate H_2O_2 leading to a failure to kill microbes properly. The CGD patients provide a clear role of Nox2 and its accessory proteins in phagocytic cells.

Since Duox isozymes were identified as thyroid NADPH oxidases producing H_2O_2 for biosynthesis of thyroid hormone (De Deken et al., 2000; Dupuy et al., 1999), it has been reported that Duox isozymes are found in the ducts of the salivary gland (Geiszt et al., 2003b), airway epithelia, rectal mucosa, and intestinal colon epithelia (Dupuy et al., 2000; El Hassani et al., 2005). Conceptually, epithelia of airway and intestinal colon are front line to contact many bacterial species and require appropriate machinery to controlling microbes. Substantial evidences indicate that both of Duox and lactoperoxidase (LPO) secreted in airway and mucosal epithelia appear to be an important role in microbial killing. LPO generates hypothiocyatnate (OSCN-) from the oxidation of SCN- by Duox-dependent H_2O_2 (Geiszt et al., 2003b). In airway cells, amount of H_2O_2 (about 90 nM) produced in exhale breath appears to be sufficient for maintaining LPO activity (Schleiss et al., 2000). ATP binding cassette (ABC) family protein, cystic fibrosis transmembrane conductance regulator (CFTR) was shown to transport Cl^- (Tabcharani et al.,

1993; Verkman et al., 2003) as well as SCN- in apical membrane of airway epithelium (Moskwa et al., 2007). It is shown that Duox-induced H_2O_2 serves as fuel for LPO-mediated hypothiocyanate production, providing robust antimicrobial defense network in epithelial cells.

Because Duox contains intracellular EF hand Ca^{2+} binding motif, agonists triggering intracellular Ca^{2+} mobilization appear to activate Duox isozymes. Extracellular ATP binds to purinergic receptor expressed on surface of airway epithelia cells and then mobilizes intracellular Ca^{2+} through classical G-protein signaling cascade, leading to H_2O_2 production followed by Duox activation on apical membrane (Forteza et al., 2005). It has recently reported that calcium-dependent Duox activity was regulated by protein kinases (Rigutto et al., 2009) and interaction with NoxA1 (Pacquelet et al., 2008). These experimental results provide an insight to regulation mechanism of Duox activity. Since genetic defect of Duox gene associates to hypothyroidsm (Moreno et al., 2002), a few clinical reports indicate correlation of Duox with lung and mucosal diseases. Knaus and collegues reported that silencing Duox gene by hypermethylation on CpG-rich promoter region results in increasing incidence of lung cancer suggesting that Duox plays an important role in mucosal homeostasis and wound healing (Luxen et al., 2008).

Receptor-mediated Nox activation

Recently, many reports have demonstrated that intracellular ROS, produced in mammalian cells in response to the activation of various receptors, serve as important second messengers in cell signaling. Cysteine residue in active center has low pK_a and is very susceptible to oxidant. Transient produced ROS oxidized sulfhydryl group (-SH) of cysteine to sulfenic (-SOH). Several reports indicate that cysteine residue of protein tyrosine phosphatase is oxidized by agonist-induced ROS and converts to inactive form leading to increasing tyrosine phosphorylation in cells. Because uncontrolled ROS generation is very toxic, the activity of Nox isozymes in cells is tightly regulated by receptor and its signaling cascades. In following part, we will discuss Nox activation and ROS generation through receptor-mediated cell signaling.

Growth factor receptor

Upon ligation of growth factor to their receptor on cell surface, the ligand stimulates receptor dimerization resulting in transactivation of intracellular protein tyrosine kinase (PTK). Tyrosine phosphorylation in COOH-terminal region by PTK serves as binding site for cytosolic signaling proteins containing Src-homology domain 2 (SH2). It activates phospholipase Cγ (PLCγ)-PKC, phophatidylinositol 3-kinase (PI3K)-Akt, and Grb2-Sos-Ras-Erk pathway. Since the novel finding of PDGF-dependent H_2O_2 production in SMC by Finkel and colleagues (Sundaresan et al., 1995), many reports demonstrated that PDGF stimulates ROS generation in various cell types such as endothelial cells, heaptocytes, pancreatic satellite cells, and fibroblast. Although the expression of Nox2 is low in the non-phagocytic cells, substantial reports showed that Nox2 and their accessory proteins are involved in ROS generation in response to PDGF in endothelial cells, smooth muscle cells, and pancreatic satellite cells. The $p47^{phox}$ was phosphorylated by PKC and translocated to membrane in response to PDGF in EC and SMC. Moreover, vascular SMC from $p47^{phox+/+}$ mice was activated and produced ROS in response to PDGF or PMA, whereas $p47^{phox-/-}$ SMCs failed to generate ROS. PI3K generates PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 from PI, PI(4)P and PI(4,5)P_2, respectively. The phosphorylated PI serves as binding site for Phox homology (PX) domain in cytosolic proteins of Nox complex and pleckstrin homology (PH) domain in RacGEF protein. Interaction of PI(3,4)P_2 or PI(3,5)P_2 with $p47^{phox}$ or NoxO1 stimulated membrane translocation and activated Nox2 or Nox1 complex. Rac is essential component for Nox activity and is regulated by Guanine nucleotide exchange factors (GEF) as Rac activator and GTPase-activating proteins (GAP) as Rac inhibitor. Activation of βPix as RacGEF through the interaction of PI(3,4,5)P_3 or PI(3,4)P_2 with PH domain contributes to stimulate Nox1 activity (Park et al., 2004). Moreover, βPix binds to COOH-terminal region of Nox1 providing a complex formation of Nox1, βPix, and NoxO1 (Park et al., 2006b). It has been reported that basic fibroblast growth factor (bFGF) stimulates PI3K-Rac pathway resulting in Nox1 activation in SMCs (Schroder et al., 2007). Insulin induced ROS production through the regulation of Nox4 expression resulting in adipocyte differenttiation and depletion of Nox4 by the transfection of Nox4 siRNA inhibited Erk phosphorylation (Mouche et al., 2007). However, the report did not provide underlying molecular mechanism between insulin signaling pathway and epigenetic regulation for Nox4 expression.

G-protein coupled receptor

G-protein coupled receptor contains seven transmembrane domains and cytosolic long carboxyl terminal region providing heterotrimeric G-proteins. G-proteins are consisted of three subunits, α, β, and γ. Alpha subunit of G protein (G_α) can bind GDP in inactive state and become exchanged GTP for GDP leading to dissociation of $G_{\beta\gamma}$ complex and in turns to active state. Binding of ligand induced conformational change of receptor resulting in the activation of G-protein. Angiotensin II (Ang II) binds directly to the angiotensin type 1 receptor (AT_1R) which belongs to the G protein-coupled receptor family. Engagement of AT_1R elicits a complex network of signaling cascades that result in short-term vascular effects such as contraction, as well as long-term effects such as cell growth, migration, and adhesion, that eventually lead to pathophysiological vascular remodeling and cardiac hypertrophy (de Gasparo et al., 2000; Spat and Hunyady, 2004). Ang II-AT_1R complex elicited a biphasic ROS production in SMCs. Early ROS production was responsible for $G_{\alpha q}$-mediated activation of phospholipase C (PLC)-β, which is followed by Ca^{2+} mobilization and activation of protein kinase C (PKC). In reconstituted cells (Nox1-NoxO1-NoxA1 or Nox2-$p47^{phox}$-$p67^{phox}$) stimulated with Ang II, Nox2 and Nox1 are activated by Ang II-AT_1R signaling cascade. However, the activity of Nox2-$p47^{phox}$-$p67^{phox}$ complex is required for the activation of PKC, PI3K and Rac1, rather than that of Nox1-NoxO1-NoxA1 complex. Second peak for ROS generation by Ang II was resulting from up-regulation of Nox1, $p22^{phox}$, and $p47^{phox}$ (Choi et al., 2008). Transcriptional factor Ets1 plays a critical for Ang II-mediated ROS generation by regulating the expression of $p47^{phox}$ (Ni et al., 2007).

Toll-like receptor

In human, 10 members of the Toll-like receptor (TLR) family have been identified and shown to be involved in innate immunity and inflammation responses. TLRs are belong to TLR/interleukin 1 receptor superfamily containing leucine-rich-repeat (LRR) domain for ligand binding site and conserved carboxyl terminal region (Toll/IL-1R, TIR domain) for recruitment of intracellular signaling molecules. Extracellular domain of TLRs recognizes pathogen-associated molecular patterns (PAMPs) on the surface of pathogens. Most of TLRs constitute homodimer formation for recognition of pathogens (Beutler, 2009; Kawai and Akira, 2010). However, TLR2 forms with heterodimer with TLR1 or TLR6. TLR2-TLR1 and TLR2-TLR6 recognize triacety-

lated and diacetylated lipoprotein, respectively. TLR4 binds to lipo-polysaccaride (LPS), an integral component of the outer membrane of Gram-negative bacteria, and TLR5 recognizes bacterial flagellin. TLR7/8 and TLR9 are involved in recognition of viral single strand RNA and bacterial DNA containing CpG motifs. Ligation of TLRs by ligand leads to stimulation of signaling networks conferring an increased expression of host defense proteins and inflammatory cytokines. Upon binding of ligand with TLRs, the cytoplasmic region of TLRs recruits adaptor protein myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor protein-inducing IFNβ (TRIF) (Baccala et al., 2007). The MyD88-dependent pathway appears to be involved in the recruitment of IL-1R associated kinase (IRAK) associated with TRAF6 leading to NF-κB activation. Activated cells by TLR3 agonist also operate MyD88-independent TRIF-dependent pathway stimulating IRF3 for INFβ production and delayed NF-κB activation through PKC, PI3-Kinase, and Akt.

It has been reported that Nox1 is involved in TLR-mediated innate immunity and host defense in gastric and colon epithelial cells (Rokutan et al., 2006). LPS from *Helicobacter pylori* stimulated ROS generation through the induction of Nox1 and NoxO1 expression in guinea pig gastric pit cells resulting from TLR4-dependent NF-κB activation pathway. LPS-dependent ROS contributed the overexpression of tumor necrosis factor α (TNFα) and cyclooxygenase in gastric pit cells suggesting that it might be involved in *Helicobacter pylori*-dependent inflammation and pathogenesis. Flagellin from *Salmonella enteritides* and recombinant flagellin (rFliC) were shown to induce ROS generation in guinea pig large intestinal epithelial cells (LIECs) and T84 human colon epithelium co-transfected with NoxO1 and NoxA1 leading to triggering inflammatory cytokine IL-8 production through TGFβ-activated kinase 1 and TGFβ-activated kinase 1-binding protein 1 and induction of Nox1 through NF-κB and INFγ. Moreover, INFγ-stimulated transcription factor appears to be involved in Nox1 expression leading to mucosal host defense and pathogenic inflammation in the colon.

It has been reported that binding of LPS to TLR4 triggers various cell signaling pathways including NF-κB activation and ROS production. LPS-induced ROS generation and NF-κB activation was mediated by a direct interaction of TLR4 with Nox4 in HEK293T cells (Park et al., 2006a). Yeast two hybrid and GST pull-down assays indicated that the carboxyl terminal region of Nox4 interactes with the cytoplasmic region of TLR4. Knockdown of Nox4 using Nox4 siRNA in HEK293T cells expressing TLR4 along with MD2 and CD14 resulted in inhibition of LPS-induced ROS generation and NF-κB activation. Moreover, it has been demonstrated that Nox4-dependent ROS generation plays an important role in LPS-induced proinflammatory cytokine production by EC and activation, leading to adhesion molecule expression. Downregulation of Nox4 in HAECs resulted in a failure to induce ROS generation and subsequent expression of intercellular adhesion molecule-1 (ICAM-1) and chemokines such as IL-8 and monocyte chemo-attractant protein-1 (MCP-1) in response to LPS. These results suggest that generated ROS from EC may play an important role in the process of atherogenesis because infiltration of blood vessel intima by leukocytes, aided by the expression of chemokines and adhesion molecules, is an initial and rate-limiting step in the development of atherosclerotic lesions. Recent report suggested that Nox4 is shown to be involved in chemokine CXCR6 expression through TLR4-Nox4-AP1 cascade (Patel et al., 2006). However, molecular connection between TLR4 and Nox4 in vascular cells is needed further studies.

TLRs also recognize endogenous ligands which are involved in inflammation process and are found in inflammation sites. The endogenous ligands appear to be damage-associated molecular patterns (DAMPs) which are derived from damage tissues and triggers inflammation cascades in injured sites. Apoptotic cells contain Oxidation of molecules generates oxidation-specific epitopes serving as danger signals to trigger inflammation reactions. It has been reported that minimally oxidized LDL (mmLDL) interacts with CD14 and induces secretion of certain cytokines through TLR4 (Miller et al., 2003; 2005). mmLDL stimulates ROS generation in macrophages via activation of TLR4, and a subsequent signaling cascade involving Syk, PLCγ, PKC and Nox2 (Bae et al., 2009). Moreover, the production and functional role of specific pro-inflammatory cytokines in response to mmLDL requires the) presence of Nox2. mmLDL stimulated intracellular reactive oxygen species (ROS) generation in macrophages through Nox2 through TLR4. ROS generation by mmLDL required the recruitment and activation of spleen tyrosine kinase (Syk) and that mmLDL also induced PLCγ1 phosphorylation and PKC membrane translocation resulting in Nox2 activation. Generation of ROS by Nox2 modulated mmLDL activation of macrophages by regulating the expression of proinflammatory cytokines IL-1β, IL-6 and RANTES. RANTES was able to stimulate migration of mouse aortic smooth muscle cells (MASMC). These data explained mechanisms by which endogenous ligands mmLDL for TLR4 can induce proatherogenic activation of macrophages. However, active molecule in mmLDL is still unclear. Oxidized form of 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (Ox-PAPC) stimulated Nox activity resulting in induction of inflammatory and sterol regulatory genes in EC (Rouhanizadeh et al., 2005).

CONCLUSIONS

While moderate levels of ROS are useful for cellular proliferation and host defense, excessive amounts of ROS can be harmful to cellular metabolism and cause unwanted events, such as damage to DNA, RNA, and proteins, and induction of apoptosis or necrosis (Valko et al., 2006). Mitochondrial superoxide is continuously produced from the mitochondrial respiratory chain and the majority of ROS are generated by leakage of electrons in the mitochondrial respiratory chain. This ROS production contributes to mitochondrial and cellular damages, and ROS play a critical role in many diseases including cancer, arthritis, aging, neurodegenerative disorders, and diabetes (Droge, 2002). Since persistent accumulation of DNA damage induced by ROS is proposed to be a major contributor of cancer and aging, the possibility of ROS as therapeutic targets in anti-cancer therapy has been frequently raised. The levels of ROS in cancer cells are close to the threshold that causes cell death and the source of ROS production in tumor cells is different from that of normal cells (Laurent et al., 2005; Szatrowski and Nathan, 1991). Therefore, Kong et al. have suggested that up-regulation of ROS by treatment with antioxidant enzyme inhibitors or ROS inducers can cause further oxidative stress that preferentially triggers tumor cell death (Kong et al., 2000).

ROS generation is tightly controlled by various proteins including mitochondrial proteins, cytochrome p450 (CYP), and Nox isozymes. Although cells regulate ROS generation for the maintenance of redox homeostasis and cellular signaling pathways, the molecular mechanism by which the proteins located in sub-cellular organelles such as mitochondria, endoplasmic reticulum, endosome and plasma membrane regulate ROS generation still remains to be elucidated. If the expression and the activation of these proteins are dysregulated in cells, normal redox signaling will be disturbed and the subcellular organelles

will sometimes face oxidative damages. Therefore, understanding and verifying mechanism of ROS generation will provide important information for drug development for diseases related to redox homeostasis.

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