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RESEARCH ARTICLE

Differential role of reactive oxygen species in the activation of mitogen-activated protein kinases and Akt by key receptors on B-lymphocytes: CD40, the B cell antigen receptor, and CXCR4

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Abstract
Background Antibodies produced by B-lymphocytes play a key role in the host defense against infection. The development, survival, and activation of B cell is regulated by multiple receptors including the B cell antigen receptor (BCR), which detects the presence of pathogens, CD40, which binds co-stimulatory molecules on activated T cells, and chemokines such as SDF-1 (CXCL12) that play key roles in B cell development and trafficking. Signaling by many receptors results in the generation of reactive oxygen species (ROS) that function as second messengers by regulating the activity of redox-sensitive kinases and phosphatases. We investigated the role of ROS in signaling by the BCR, CD40, and CXCR4, the receptor for SDF-1. We focused on activation of ERK, JNK, p38, and Akt, kinases that regulate multiple processes including cell survival, proliferation, and migration.
Results Using the anti-oxidants *N*-acetyl *L*-cysteine (NAC) and ebselen to deplete intracellular ROS, we identified a differential requirement for ROS in the activation of ERK, JNK, p38, and Akt by these receptors. We found that CD40 activated JNK, p38, and Akt via redox-dependent pathways that were sensitive to ROS depletion by NAC and ebselen. In contrast, BCR-induced activation of ERK, JNK, p38, and Akt was not affected by ROS depletion. We also found that CXCR4-induced Akt activation was ROS-dependent even though activation of the ERK, JNK, and p38 MAP kinases by CXCR4 occurred via ROS-independent pathways.
Conclusion The differential requirement for ROS in the activation of ERK, JNK, p38, and Akt by the BCR, CD40, and CXCR4 likely reflects the multiplicity of upstream activators for each of these kinases, only some of which may be regulated in a redox-dependent manner. These findings support the idea that ROS are important second messengers in B cells and suggest that oxidants or anti-oxidants could be used to modulate B cell activation.

Keywords B-lymphocytes · Reactive oxygen species · CD40 · BCR · CXCR4 · MAP kinases · Akt

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Abbreviations

BCR	B cell antigen receptor
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
NAC	*N*-acetyl *L*-cysteine
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1
TBS	Tris-buffered saline
TNF	Tumor necrosis factor

Background

B-lymphocytes play a key role in host defenses against infection by producing antibodies that help eliminate pathogens and neutralize secreted toxins. The development, selection, survival, activation, and proliferation of B-lymphocytes, as well as the differentiation of B cells into antibody-producing plasma cells, is regulated by antigens, T cell-derived co-stimulatory signals, and chemokines (Bishop et al. 2003). Antigen-induced signaling via the B cell antigen receptor (BCR) mediates the elimination or silencing of self-reactive B cells as well as the activation of B cells that recognize foreign antigens (Niiro and Clark 2002; Gold 2002). T cells deliver essential co-stimulatory signals to B cells via CD40, a tumor necrosis factor (TNF) family receptor that activates B cells and prevents BCR-induced tolerance (anergy) or apoptosis (Bishop and Hostager 2003; Santos-Argumedo et al. 1994). A variety of chemokines regulate B cell development and activation by directing the trafficking and adhesion of B cells. In particular, the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) is a survival factor for B cell progenitors, retains pro-B cells in the bone marrow where they develop (Nagasawa et al. 1996; Ma et al. 1998), contributes to the entry of mature B cells into lymphoid organs via high endothelial venules (Miyasaka and Tanaka 2004), and directs plasma cells to the bone marrow (Hargreaves et al. 2001), a niche in which they can survive and produce antibodies for long periods of time.

The ERK, JNK, and p38 mitogen-activated protein kinases (MAPKs) are key signaling intermediates by which many receptors regulate cell growth and survival, apoptosis, proliferation, and differentiation (Yoon and Seger 2006; Karin and Gallagher 2005; Zarubin and Han 2005). In addition to cytosolic proteins that regulate diverse processes, many MAPK substrates are either transcription factors or kinases that phosphorylate transcription factors. In B cells, the BCR and CD40 activate all three families of MAPKs, although to different extents (Sutherland et al. 1996; Purkerson and Parker 1998; Sakata et al. 1995; Berberich et al. 1996). For example, in the WEHI-231 B lymphoma cell line, the BCR activates ERK to a much greater extent than JNK or p38 while CD40 strongly activates JNK and p38 but causes only marginal ERK activation (Sutherland et al. 1996). CXCR4, the receptor for SDF-1, transiently activates both ERK and JNK in B cells (Ganju et al. 1998; McLeod et al. 2002; Ortolano et al. 2006) and JNK activation is important for SDF-1-induced B cell migration (Ortolano et al. 2006).

MAPK signaling plays an important role in BCR- and CD40-induced survival, activation and differentiation in both normal and malignant B cells. In murine splenic B cells, ERK activation is important for BCR-induced proliferation and for BCR-induced upregulation of the Egr-1 transcription factor, the CD44 adhesion molecule, and the CD69 activation marker (Richards et al. 2001). Activation of ERK by the BCR also promotes the phosphorylation and degradation of Bcl-6 (Niu et al. 1998), a transcriptional repressor whose elimination is required for B cells to differentiate into plasma cells. Both p38 and JNK mediate CD40-induced B cell activation. The p38 MAP kinases play a major role in CD40-induced gene expression in B cells (Craxton et al. 1998; Dadgostar et al. 2002) and are required for CD40-induced B cell proliferation (Craxton et al. 1998). JNK activation is required for CD40-mediated IgE class switching (Jabara and Geha 2005). In contrast, activation of JNK and p38 by the BCR promotes the apoptosis of B lymphoma cells lines (Graves et al. 1998; Takada et al. 2001; Swart and Chiles 2000).

Another important kinase that is activated in B cells by the BCR (Gold et al. 1999), CD40 (Andjelic et al. 2000), and CXCR4 (McLeod et al. 2002; Ortolano et al. 2006) is Akt/protein kinase B, a downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway. Akt is a central regulator of cell survival (Scheid and Woodgett 2001; Song et al. 2005) and Akt activation is required for the survival and proliferation of normal murine splenic B cells (Aiba et al. 2006), the WEHI-231 murine B cell lymphoma (Banerji et al. 2001), and many types of human B cell tumors including mantle cell lymphomas and multiple myelomas (Hsu et al. 2002; Rudelius et al. 2006). Akt promotes cell growth and survival by phosphorylating and inhibiting multiple proteins that promote cell cycle arrest and apoptosis including FKHRL-1, a transcription factor that induces the expression of cell cycle arrest proteins, $p27^{kip1}$, a negative regulator of cell cycle progression, BAD, a pro-apoptotic member of the Bcl-2 family, and the GSK-3 kinase, a negative regulator of protein translation and cell cycle progression (Scheid and Woodgett 2001; Song et al. 2005). Akt also increases protein translation through the tuberin/Rheb/mTOR pathway (Manning and Cantley 2003), contributes to the activation of NF-κB (Kane et al. 1999), and plays a role in cell migration (Enomoto et al. 2005).

Upon ligand binding, a wide variety of receptors including receptor tyrosine kinases, antigen receptors, TNF receptor family members, Toll-like receptors, and G protein-coupled receptors induce the generation of intracellular reactive oxygen species (ROS) such as hydrogen peroxide and superoxide (Sundaresan et al. 1995; Fang et al. 1995; Devadas et al. 2002; Lo and Cruz 1995; Lee and Koretzky 1998; Ha and Lee 2004a; Matsuzawa et al. 2005; Griendling and Ushio-Fukai 2000). Importantly, a

large body of work indicates that these ROS can act as second messengers. Many signaling molecules, particularly kinases and phosphatases, are sensitive to the redox state of their environment (Reth 2002; Tonks 2005; Adler et al. 1999). Treating cells with anti-oxidants that lower the levels of intracellular ROS can inhibit receptor-induced activation of JNK (Lee and Koretzky 1998; Lo et al. 1996; Viedt et al. 2000), p38 (Griendling and Ushio-Fukai 2000; Viedt et al. 2000; Asehnoune et al. 2004; Ushio-Fukai et al. 1998), ERK (Devadas et al. 2002; Asehnoune et al. 2004; Daou and Srivasta 2004; Tanaka et al. 2001; Hannken et al. 2000), and Akt (Asehnoune et al. 2004; Daou and Srivasta 2004; Ha et al. 2004b; Gorin et al. 2001; Ushio-Fukai et al. 1999). However, there are many examples in which receptor-induced activation of these kinases is unaffected by ROS scavengers (Ha et al. 2004b; Harfouche et al. 2005; Li and Malik 2005). Whether or not the MAP kinases and Akt are activated in a ROS-dependent manner depends on both the receptor and the cellular context. Activation of a kinase by a particular receptor can be ROS-dependent in one cell type but ROS-independent in another cell type. Also, within a single cell one receptor may activate a MAP kinase or Akt in a ROS-dependent manner while a different receptor activates the same kinase in a ROS-independent manner. This differential requirement for ROS likely reflects the large number of different upstream activators for each of the MAP kinases, as well as the multiple PI3K isoforms that can activate Akt. Only a subset of these upstream activators of the MAP kinases and Akt may be redox-dependent.

Although the BCR (Fang et al. 1995; Hamano et al. 2002; Singh et al. 2005) and CD40 (Lee and Koretzky 1998; Ha and Lee 2004a; Laxmanan et al. 2005) stimulate ROS generation when engaged, the role of ROS in B cell signaling and in B cell activation is not completely understood. Lee and colleagues have shown that ROS are required for CD40-induced activation of both JNK and p38 (Lee and Koretzky 1998; Ha and Lee 2004a). The role of ROS in CD40-induced activation of Akt has not been investigated. Although little is known about the role of ROS in the activation of specific signaling pathways by the BCR, the inhibition of protein tyrosine phosphatases by ROS plays an important role in the initiation and amplification of BCR signaling by Src family tyrosine kinases and the Syk tyrosine kinase (Singh et al. 2005; Rolli et al. 2002). Consistent with a role for ROS in BCR-induced B cell activation, treating murine splenic B cells with anti-oxidants inhibits BCR-induced proliferation (Fedyk and Phillips 1994). The role of ROS generation in CXCR4 signaling in B cells has not been investigated. In smooth muscle cells, SDF-1-induced production of tissue factor is blocked by anti-oxidants (Schecter et al. 2001), suggesting that CXCR4 might stimulate ROS production. Moreover, other G protein-coupled receptors that bind angiotensin II receptor and monocyte chemotactic protein-1 induce ROS formation (Griendling and Ushio-Fukai 2000; Lo et al. 2005).

To more fully understand the role of ROS in B cells, we investigated the requirement for ROS in the activation of MAPKs and Akt by the BCR, CD40, and CXCR4. By treating WEHI-231 B lymphoma cells with the membrane-permeable anti-oxidants *N*-acetyl *L*-cysteine (NAC) and ebselen, we reveal a differential role for ROS in coupling these receptors to the activation of JNK, p38, ERK, and Akt.

Results

Differential requirement for ROS in the activation of MAP kinases by CD40, the BCR, and CXCR4

ROS have been shown to be important for CD40-induced activation of the JNK and p38 MAP kinases in B cells (Lee and Koretzky 1998; Ha and Lee 2004a). Since signaling by the BCR and G protein-coupled receptors like CXCR4 also leads to the generation of intracellular ROS (Fang et al. 1995; Griendling and Ushio-Fukai 2000; Hamano et al. 2002; Singh et al. 2005; Lo et al. 2005), we investigated whether ROS are involved in the activation of JNK and p38 by the BCR and CXCR4. To test this, we pretreated WEHI-231 B lymphoma cells with two different compounds that reduce intracellular ROS levels, NAC and ebselen. NAC is a ROS scavenger (Aruoma et al. 1989) that also increases intracellular levels of reduced glutathione (Faruqi et al. 1997), the major endogenous anti-oxidant in cells. Ebselen is a glutathione peroxidase mimic that uses reduced glutathione to convert H_2O_2 to water and oxygen (Sies 1993). The WEHI-231 B lymphoma cell line was chosen as a model system since it has been extensively characterized in terms of the signaling and cellular responses induced by CD40, the BCR, and CXCR4 (Santos-Argumedo et al. 1994; Sutherland et al. 1996; McLeod et al. 2002; Richards et al. 2001; Gold et al. 1999; Lee and Koretzky 1998). Moreover, it resembles an immature/transitional B cell and has been widely used to study BCR-induced apoptosis, CD40-mediated survival, and SDF-1-induced migration. Finally, Lee et al. also used WEHI-231 cells to study the role of ROS in CD40 signaling (Lee and Koretzky 1998; Ha and Lee 2004a).

To assess the activation or JNK and p38, which are often activated in a coordinated manner, we probed cell lysates with antibodies that specifically detect the phosphorylation of these kinases on threonine-X-tyrosine motifs in their activation loops, modifications that are essential for their

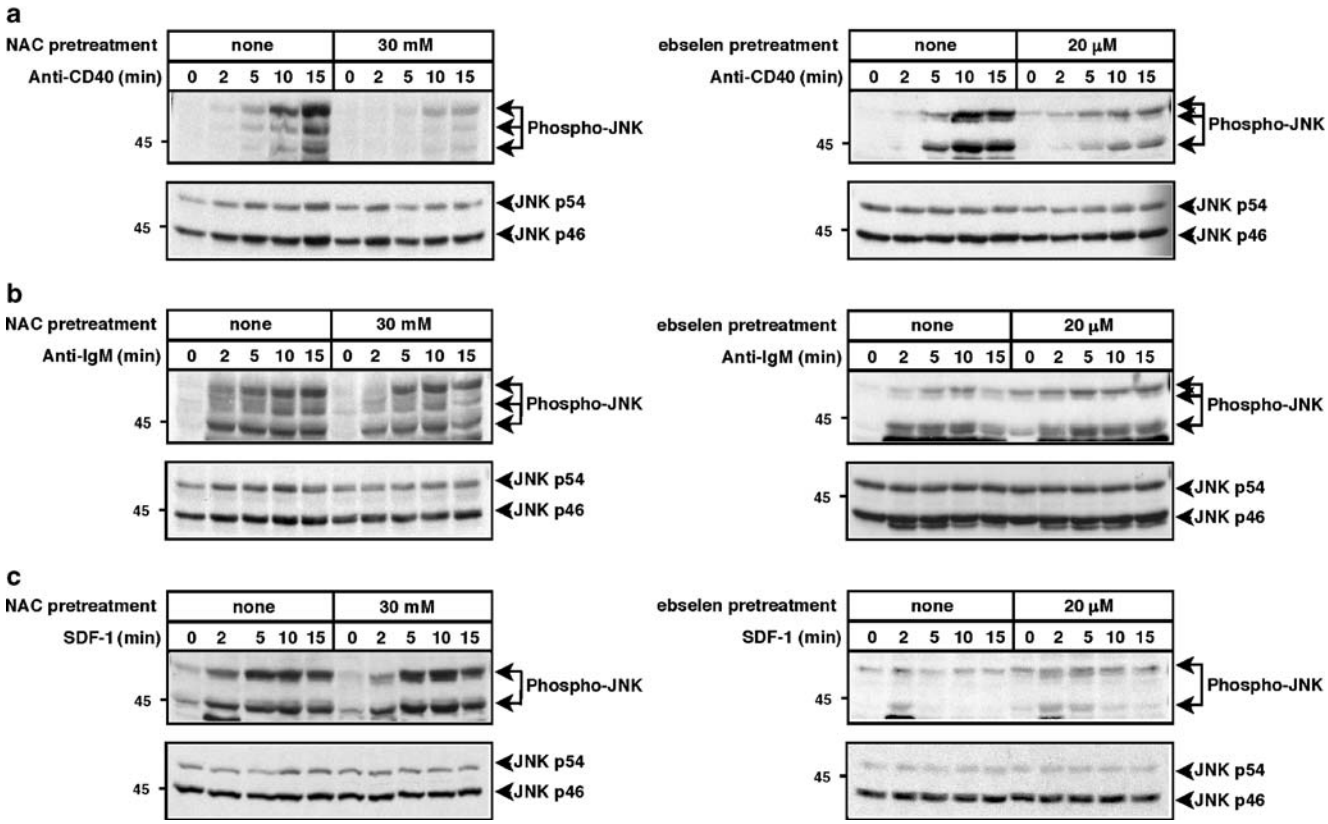

Fig. 1 Depletion of ROS with NAC or ebselen inhibits anti-CD40-induced JNK activation but does not inhibit anti-IgM- or SDF-1-induced JNK activation. WEHI-231 cells were pretreated with 30 mM NAC for 2 h or with 20 μM ebselen for 1 h and then stimulated for the indicated times with **a** 10 μg/ml of the 1C10 anti-CD40 monoclonal antibody, **b** 30 μg/ml anti-IgM or **c** 100 ng/ml SDF-1. When cells were pretreated with ebselen, the control cells were incubated with an equivalent volume of DMSO. Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with a phospho-specific antibody that recognizes the activated form of JNK (*upper panels*). The blots were then stripped and reprobed with anti-JNK antibodies to show that equivalent amounts of JNK were present in each sample (*lower panels*). Molecular mass markers (in kDa) are shown to the left. For each panel, similar results were obtained in at least three independent experiments

activation. Figures 1 and 2 show that CD40, the BCR, and CXCR4 all activated JNK and p38, with maximal responses usually occurring at 5–15 min. Reducing ROS levels by pre-treating WEHI-231 cells with either 30 mM NAC or 20 μM ebselen substantially reduced anti-CD40-induced phosphorylation of JNK and p38 (Figs. 1a, 2a), as had been shown previously by Lee et al. (Lee and Koretzky 1998; Ha and Lee 2004a). ROS depletion reduced CD40-induced JNK phosphorylation to a greater extent than p38 phosphorylation. In contrast to CD40-induced activation of JNK and p38, reducing ROS levels had very little effect on anti-IgM- or SDF-1-induced phosphorylation of JNK (Fig. 1b,c) or p38 (Fig. 2b,c). Thus, CD40 activates JNK and p38 via pathways that are largely ROS-dependent whereas the BCR and CXCR4 activate JNK and p38 via pathways that are relatively insensitive to ROS depletion. The inability of NAC and ebselen to suppress BCR-induced activation of JNK and p38 shows that these compounds were not toxic to the cells at the concentrations used and that they did not suppress all signaling reactions in a non-specific manner.

A similar approach was used to assess whether ROS play a role in activation of the ERK1 and ERK2 MAP kinases in WEHI-231 cells. Figure 3 shows that anti-IgM caused sustained ERK phosphorylation while SDF-1 caused strong initial ERK phosphorylation, followed by a rapid decrease. We have previously shown that CD40 causes little or no ERK activation in WEHI-231 cells (Sutherland et al. 1996). Neither NAC nor ebselen significantly reduced anti-IgM- or SDF-1-induced ERK phosphorylation (Fig. 3), indicating that the BCR and CXCR4 do not use ROS-dependent pathways to activate ERK. Thus, CD40-induced activation of JNK and p38 is ROS-dependent whereas BCR- and CXCR4-induced activation of ERK, JNK, and p38 are relatively insensitive to ROS depletion.

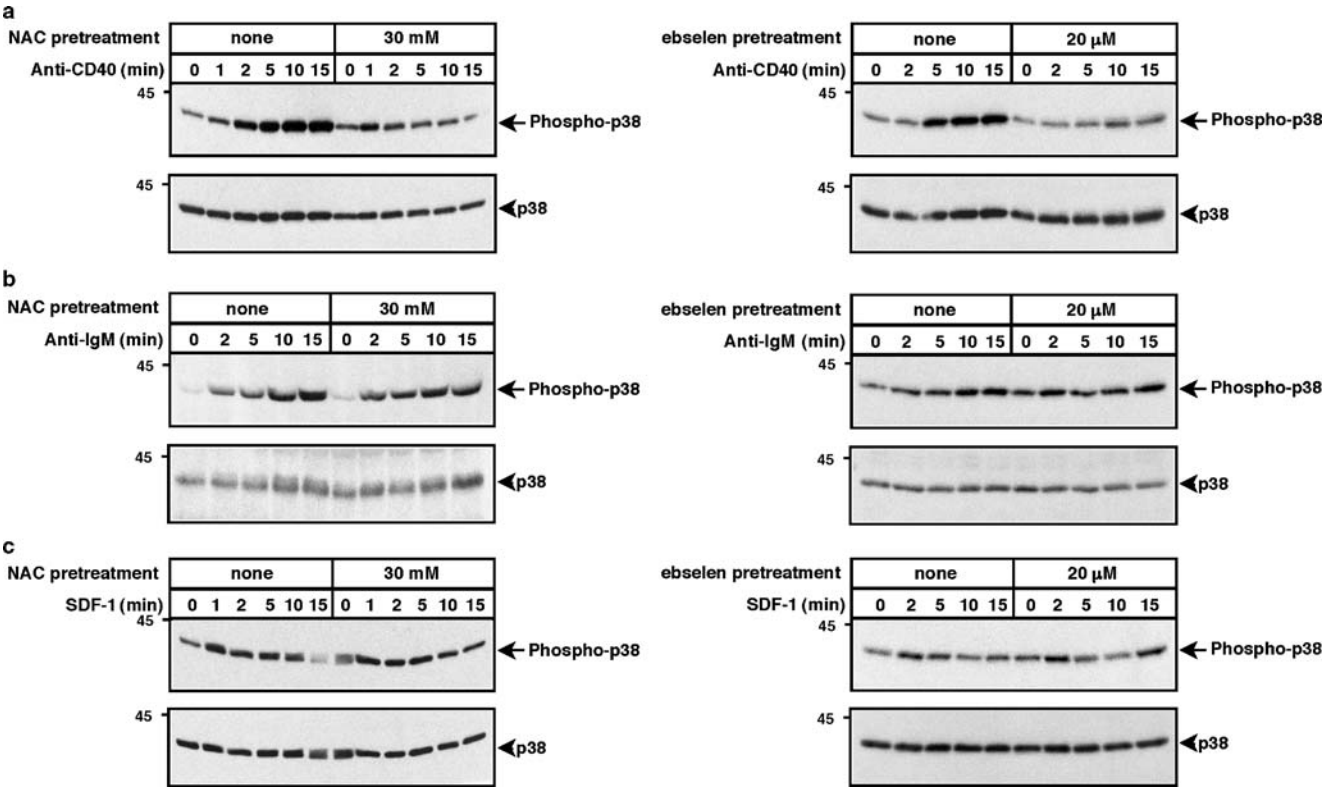

Fig. 2 Depletion of ROS with NAC or ebselen reduces anti-CD40-induced p38 activation but does not inhibit anti-IgM- or SDF-1-induced p38 activation. WEHI-231 cells were pretreated with 30 mM NAC for 2 h or with 20 μM ebselen for 1 h and then stimulated for the indicated times with **a** 10 μg/ml of the 1C10 anti-CD40 monoclonal antibody, **b** 30 μg/ml anti-IgM or **c** 100 ng/ml SDF-1. When cells were pretreated with ebselen, the control cells were incubated with an equivalent volume of DMSO. Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with a phospho-specific antibody that recognizes the activated form of p38 (*upper panels*). The blots were then stripped and reprobed with anti-p38 antibodies to show that equivalent amounts of p38 were present in each sample (*lower panels*). Molecular mass markers (in kDa) are shown to the left. For each panel, similar results were obtained in at least three independent experiments

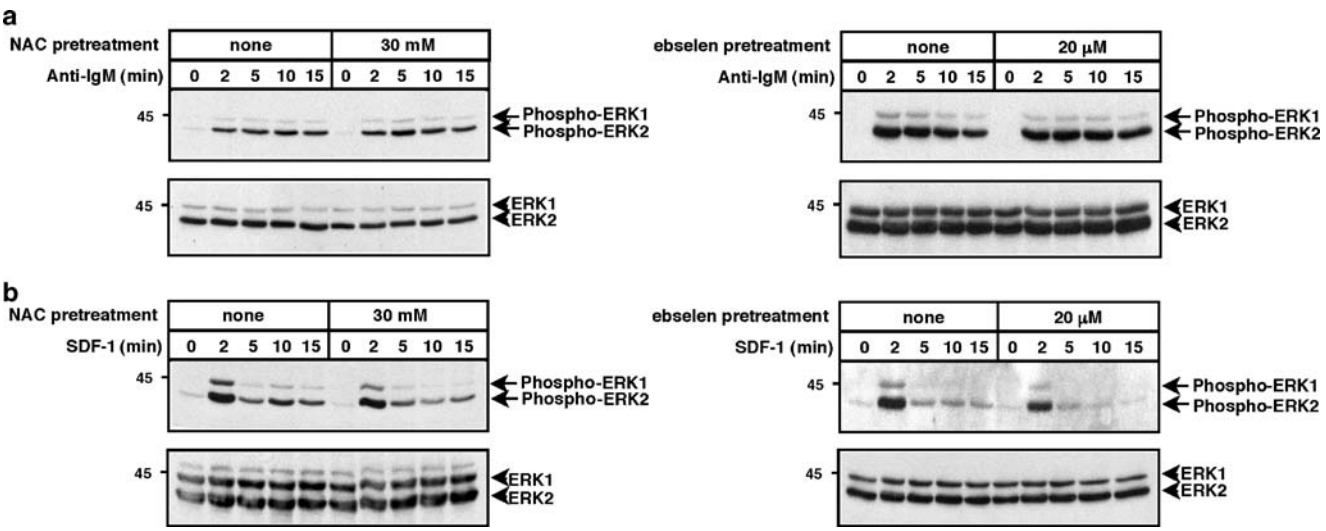

Fig. 3 Depletion of ROS with NAC or ebselen does not inhibit anti-IgM- or SDF-1-induced ERK activation. WEHI-231 cells were pretreated with 30 mM NAC for 2 h or with 20 μM ebselen for 1 h and then stimulated for the indicated times with **a** 30 μg/ml anti-IgM or **b** 100 ng/ml SDF-1. When cells were pretreated with ebselen, the control cells were incubated with an equivalent volume of DMSO. Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with a phospho-specific antibody that recognizes the activated forms of ERK1 and ERK2 (*upper panels*). The blots were then stripped and reprobed with anti-ERK1/2 antibodies to show that equivalent amounts of ERK1/2 were present in each sample (*lower panels*). Molecular mass markers (in kDa) are shown to the left. For each panel, similar results were obtained in at least three independent experiments

Differential requirement for ROS in the activation of Akt by CD40, the BCR, and CXCR4

Akt is a critical regulator of B cell survival and has also been implicated in cell migration. Previous reports have indicated that Akt activation is dependent on ROS in osteoclasts and vascular smooth muscle cells (Daou and Srivasta 2004; Ha et al. 2004b; Gorin et al. 2001; Ushio-Fukai et al. 1999). Moreover, oxidative stress can induce Akt activation in the DT40 chicken B cell line (Qin and Chock 2003). Thus we investigated whether ROS are required for the BCR, CD40, and CXCR4 to activate Akt in B cells. As a readout for Akt activation we assessed the phosphorylation of Akt on serine 473, an essential step in Akt activation. Figure 4a shows that stimulating WEHI-231 cells with 300 μM H_2O_2 induced the phosphorylation of Akt on Ser473 and that this response to H_2O_2 could be completely blocked by pre-treating the cells with NAC. Thus activation of Akt in WEHI-231 cells can be regulated in a redox-dependent manner. Indeed, both SDF-1- and anti-CD40-induced activation of Akt was substantially reduced when ROS were depleted by pretreating the cells with either NAC or ebselen (Figs. 4b,d). In contrast, anti-IgM-stimulated Akt phosphorylation was unaffected by ROS depletion (Fig. 4c). Thus, CD40 and CXCR4 activate Akt via ROS-dependent pathways whereas the BCR activates Akt via a pathway that is largely ROS-independent.

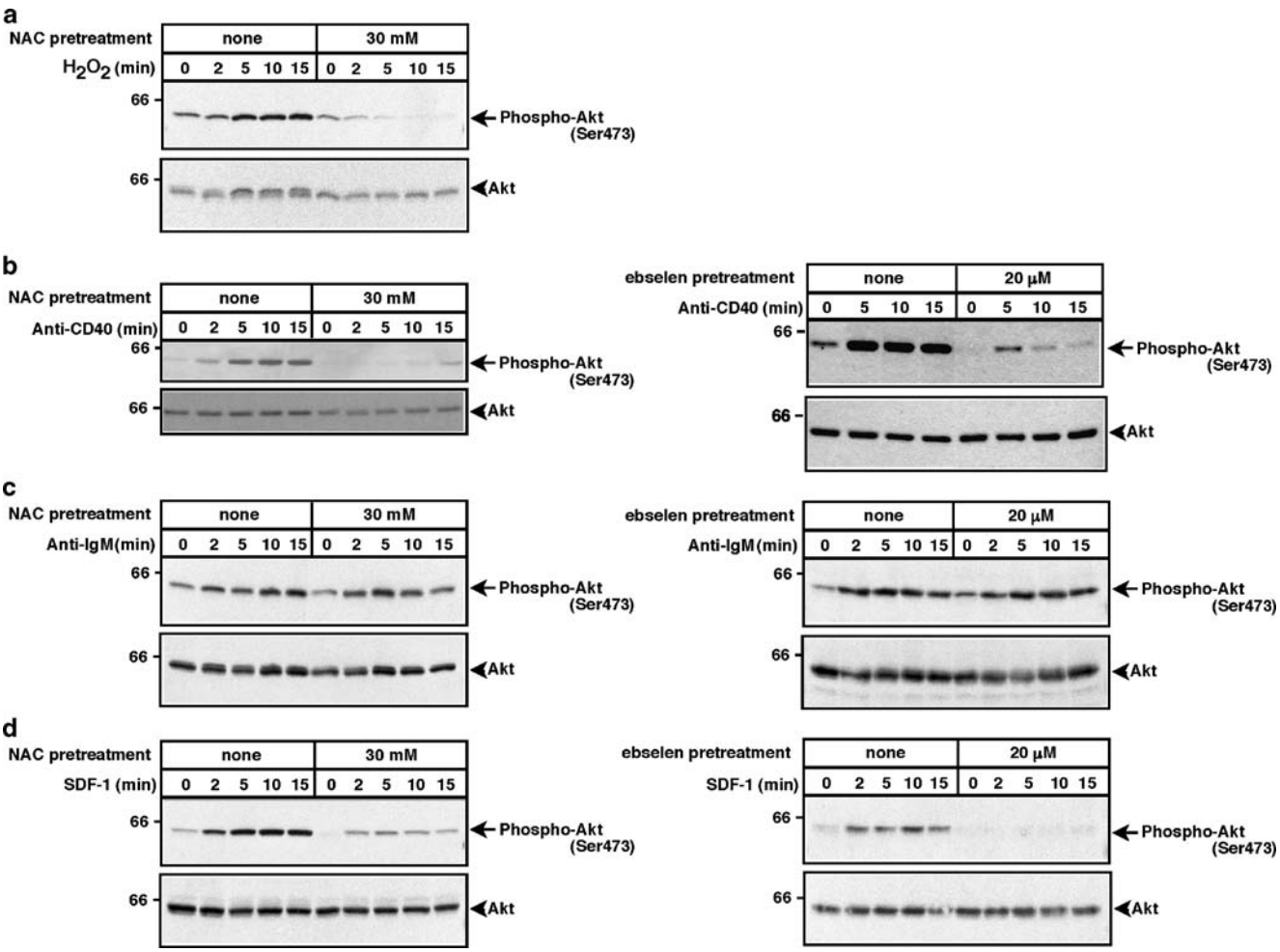

Fig. 4 Depletion of ROS with NAC or ebselen inhibits H_2O_2-, anti-CD40-, and SDF-1-induced Akt activation but does not inhibit anti-IgM-induced Akt activation. WEHI-231 cells were pretreated with 30 mM NAC for 2 h or with 20 μM ebselen for 1 h and then stimulated for the indicated times with **a** 300 μM H_2O_2, **b** 10 μg/ml of the 1C10 anti-CD40 monoclonal antibody, **c** 30 μg/ml anti-IgM or **d** 100 ng/ml SDF-1. When cells were pretreated with ebselen, the control cells were incubated with an equivalent volume of DMSO. Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with a phospho-specific antibody that recognizes the activated form of Akt, which is phosphorylated on serine 473 (*upper panels*). The blots were then stripped and reprobed with anti-Akt antibodies to show that equivalent amounts of Akt were present in each sample (*lower panels*). Molecular mass markers (in kDa) are shown to the left. For each panel, similar results were obtained in at least two independent experiments

Discussion

In this report we demonstrate a differential role for ROS in the activation of the MAP kinases and Akt by the BCR, CD40, and CXCR4 (Fig. 5). We found that activation of ERK, JNK, p38, and Akt by the BCR was relatively insensitive to ROS depletion and therefore involves signaling intermediates that are largely ROS-independent. In contrast, CD40 activates JNK, p38, and Akt for the most part via ROS-dependent signaling pathways that are sensitive to ROS depletion by either NAC or ebselen. Lee et al. had previously shown that the activation of JNK and p38 by CD40 is dependent on ROS (Lee and Koretzky 1998; Ha and Lee 2004a), but this is the first report that CD40-induced Akt activation is dependent on ROS. The role of ROS in CXCR4 signaling in B cells had not been investigated previously and we now show that CXCR4 activates the MAP kinases via ROS-independent pathways but that its ability to activate Akt is dependent on ROS.

MAPK activation involves GTPase-regulated kinase cascades. For ERK, receptor-induced conversion of Ras to its active GTP-bound form promotes the activation of the Raf-1 kinase (a MAPK kinase kinase or MAP3K), which in turn phosphorylates the MEK1 and MEK2 kinases (MAPK kinases or MAP2Ks). MEK1 and MEK2 then phosphorylate and activate ERK1 and ERK2. Although ERK is activated in a ROS-dependent manner by some receptors (Daou and Srivasta 2004; Hannken et al. 2000), particularly the G protein-coupled receptor for angiotensin II (Hannken et al. 2000), we found that both CXCR4- and BCR-induced ERK activation were ROS-independent in WEHI-231 cells.

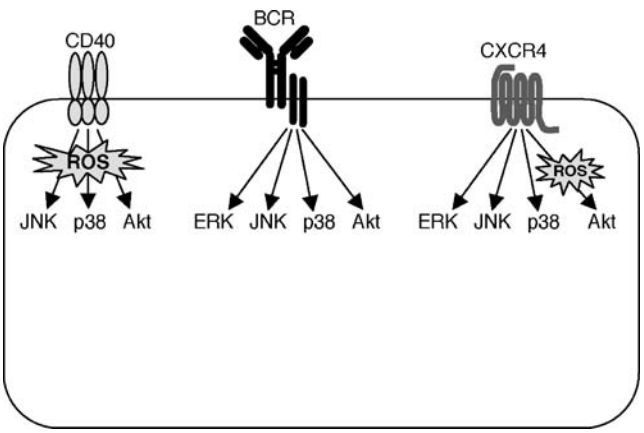

Fig. 5 Differential role of ROS in the activation of MAP kinases and Akt by the BCR, CD40, and CXCR4. Activation of ERK, JNK, p38, and Akt by the BCR is relatively insensitive to ROS depletion and involves signaling intermediates that are ROS-independent. In contrast, CD40 activates JNK, p38, and Akt via signaling pathways that are largely ROS-dependent. CXCR4 activates the MAP kinases via ROS-independent pathways but its ability to activate Akt is dependent on ROS

The kinase cascades that lead to activation of JNK and p38 are regulated by the Rac1 GTPase, which promotes the activation of MAP3Ks that coordinately activate the MKK4, MKK7, MKK3, and MKK6 MAP2Ks (Ichijo 1999). MKK4 and MKK7 activate JNK while MKK3 and MKK6 activate p38. A number of MAP3Ks including TAK1, the mixed lineage kinases (MLKs), MEKK1-4, and ASK1 can activate the JNK and p38 pathways. Each of these MAP3Ks may couple different classes of receptors or environmental stresses (e.g. oxidative stress) to the MAP2Ks that regulate JNK and p38. The possibility that some of these MAP3Ks are redox-sensitive while others are not could account for ROS-dependent versus ROS-independent activation of JNK and p38. While the redox dependence of each of the MAP3Ks upstream of JNK and p38 has not been investigated, activation of the ASK1 MAP3K is clearly dependent on ROS (Saitoh et al. 1998; Liu et al. 2000). In resting cells, thioredoxin binds to ASK1 and keeps it in an inhibited state. The generation of ROS causes thioredoxin to dissociate from ASK1, allowing ASK1 to dimerize and become activated by transphosphorylation. Once ASK1 is released from thioredoxin, it can also bind to the TRAF2, TRAF3, and TRAF6 adaptor proteins (Ha and Lee 2004a; Matsuzawa et al. 2005; Liu et al. 2000) that associate with TNF receptor family members such as CD40. Whether ASK1 is involved in the ROS-dependent activation of JNK and p38 by CD40 remains to be determined. Using a phospho-specific antibody directed against the activated form of ASK1, we were unable to detect CD40-induced ASK1 phosphorylation in WEHI-231 cells (data not shown).

While CD40 induced JNK and p38 activation in a largely ROS-dependent manner in WEHI-231 cells, we found that BCR- and CXCR4-induced activation of JNK and p38 was ROS-independent in these cells, indicating that the BCR and CXCR4 activate JNK and p38 via redox-insensitive MAP3Ks. Multiple scaffolding proteins that organize MAP3K/MAP2K/MAPK signaling modules have been identified including the JIP proteins and MEKK1 (Morrison and Davis 2003). One such scaffolding protein may couple CD40 to JNK and p38 signaling complexes that contain a redox-sensitive MAP3K such as ASK1 while different scaffolding proteins may connect the BCR and CXCR4 to JNK and p38 signaling complexes that contain a redox-insensitive MAP3K.

We also found that CD40- and CXCR4-induced Akt activation was dependent on ROS while BCR-induced activation of Akt was not inhibited by anti-oxidants. The activation of Akt depends on production of the membrane lipid phosphatidylinositol 3,4,5-trisphosphate

(PIP_3) by PI3K. PIP_3 recruits Akt to the plasma membrane where it can be activated by being phosphorylated on threonine 308 and serine 473. There are multiple isoforms of the p110 catalytic subunit of PI3K and it is possible that there are redox-sensitive and redox-insensitive p110 isoforms that would mediate ROS-dependent versus ROS-independent Akt activation. H_2O_2-induced Akt phosphorylation in the DT40 chicken B cell line can be blocked wortmannin, a PI3K inhibitor that acts on the p110 catalytic subunit (Qin and Chock 2003). This is consistent with the idea that at least one of the PI3K p110 catalytic subunits can be activated in a redox-dependent manner. Knockout mouse studies, as well as the use of a p110δ-specific inhibitor have shown that BCR-induced Akt phosphorylation is largely dependent on the p110δ isoform of the PI3K catalytic subunit (Clayton et al. 2002; Bilancio et al. 2006). Thus our finding that the BCR activates Akt in a ROS-independent manner suggests that p110δ is not a redox-regulated enzyme. The p110 isoforms that mediate ROS-dependent activation of Akt by CD40 and CXCR4 have not been identified.

ROS generation appears to be a central event in CD40 signaling as it is required for CD40 to activate JNK, p38, NF-κB (Lee and Koretzky 1998; Ha and Lee 2004a), and Akt (Fig. 4) in B cells. NF-κB activation is essential for CD40 to promote B cell survival and to overcome BCR-induced apoptotic signals in WEHI-231 cells (Schauer et al. 1996). Andjelic et al. (2000) showed that CD40-induced NF-κB activation depends on PI3K and that the PI3K/Akt pathway also links CD40 to additional pro-survival events including downregulation of the $p27^{kip}$ cell cycle inhibitor and induction of Bcl-xL. Thus, by mediating CD40-induced activation of both NF-κB and the PI3K/Akt pathway, ROS may play a central role in the ability of CD40 to promote B cell survival.

The generation of ROS by CD40 involves the Rac1 GTPase and the NADPH oxidase complex (Ha and Lee 2004a). Activated Rac1 promotes the assembly of an NADPH oxidase complex at the plasma membrane, which then transfers electrons to molecular oxygen to generate superoxide. CD40 engagement has been shown to induce the binding of the $p40^{phox}$ subunit of NADPH oxidase to the CD40-associated adaptor protein TRAF3 (Ha and Lee 2004a). Consistent with the idea that the NADPH oxidase complex is involved in ROS-dependent signaling by CD40, pre-treating WEHI-231 cells with the NADPH oxidase inhibitor diphenyleneiodonium chloride inhibits CD40-induced activation of p38 (Ha and Lee 2004a).

The mechanisms by which the BCR and CXCR4 induce ROS generation in B cells are not known. Even though the BCR and CXCR4 activate Rac1 in B cells (McLeod et al. 2002; Grill and Schrader 2002), we found that these receptors activate JNK and p38 in a ROS-independent manner. Rac1 activation has been implicated in BCR-induced activation of JNK and p38 (Hashimoto et al. 1998) but the role of Rac1 in this process may be to activate a redox-insensitive MAP3K, rather than generating ROS.

Although BCR-induced activation of the MAP kinases and Akt was not inhibited by anti-oxidant concentrations (30 mM NAC) that effectively inhibited CD40-induced activation of these kinases, ROS have been reported to play a role in the initiation of BCR signaling (Singh et al. 2005; Rolli et al. 2002). Our results suggest that BCR signaling is more resistant to anti-oxidant-induced inhibition than CD40 signaling.

Conclusions

In this report we have defined the ROS dependency of several key signaling pathways activated by three important receptors on B cells, the BCR, CD40, and CXCR4 (Fig. 5). In addition to demonstrating differential requirements for ROS in the activation of JNK, p38, and Akt by these receptors, we show for the first time that both CD40- and CXCR4-induced activation of Akt is dependent on ROS. This suggests that ROS generation could contribute to the Akt-dependent pro-survival effects of CXCR4 signaling in B cell progenitors and CD40 signaling in mature B cells. A relationship between ROS generation and B cell survival also suggests that abnormally elevated levels of intracellular ROS could contribute to excessive B cell activation or to B cell malignancies. Indeed, in patients with diffuse large B cell lymphomas, the worst prognoses correlate with decreased expression of genes encoding anti-oxidant enzymes such as catalase (Tome et al. 2005).

Materials and methods

Antibodies and chemokines

The 1C10 anti-mouse CD40 monoclonal antibody (Santos-Argumedo et al. 1994) was purified from hybridoma culture supernatant using protein G-Sepharose. Goat anti-mouse IgM antibodies (μ-chain-specific) were obtained from Jackson Immunoresearch (West Grove, PA), the 4G10 anti-phosphotyrosine monoclonal antibody was from Upstate (Charlottesville, VA) and recombinant murine SDF-1α was from R&D Systems (Minneapolis, MN). NAC was purchased from Sigma-Aldrich (St. Louis, MO) and ebselen was obtained from Calbiochem (La Jolla, CA). Phospho-specific antibodies that recognize the

activated forms of JNK, p38, ERK, and Akt, as well as antibodies against the non-phosphorylated forms of these kinases were purchased from Cell Signaling Technologies (Beverly, MA), with the exception of the anti-ERK antibody, which was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell stimulation, preparation of cell extracts, and SDS-PAGE

WEHI-231 B lymphoma cells were obtained from ATCC (Manassas, VA). Early passage cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 15 U/ml penicillin and 50 μg/ml streptomycin. To reduce basal signaling, the cells were cultured overnight in medium containing 1% fetal calf serum. Cells were washed once and resuspended to 10^7/ml in modified HEPES-buffered saline (25 mM sodium HEPES, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mg/ml glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol). The cells were pretreated with 30 mM NAC for 2 h at 37°C or with 20 μM ebselen or an equivalent volume of DMSO for 1 h. The cells were then stimulated at 37°C with 10 μg/ml of the 1C10 anti-CD40 monoclonal antibody, 30 μg/ml goat anti-mouse IgM antibodies, 100 ng/ml SDF-1, or 300 μM H_2O_2. Reactions were terminated by adding cold PBS, rapidly pelleting the cells, washing once with PBS, and then solubilizing the cells in RIPA buffer (30 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 1 μg/ml microcystin-LR [Biomol, Plymouth Meeting, PA], 20 mM NaF, 1 mM Na_3MoO_4, 10 μg/ml leupeptin). After 10 min on ice, the samples were centrifuged at 14,000 rpm for 15 min to remove detergent-insoluble material. Protein concentrations were determined using the BCA Protein Assay (Pierce Chemical Company, Rockford, IL). Total cell extracts (35 μg protein for p38 and JNK; 20 μg protein for Akt and ERK) were separated by SDS-PAGE and transferred to nitrocellulose. Prior to immunoblotting, Ponceau S staining was used to show that equivalent amounts of cell proteins were loaded in each lane.

Immunoblotting

Filters were blocked with TBS (10 mM Tris–HCl, pH 8, 150 mM NaCl) containing 0.1% Tween-20 and 5% (w/v) milk powder. The blots were then probed with phospho-specific antibodies for the active forms of ERK, JNK, p38, or Akt. Blots were stripped using TBS, pH 2 and then reprobed with the corresponding antibodies to ERK, JNK, p38, or Akt. Antibodies were diluted in TBS plus 1% (w/v) bovine serum albumin. Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada).

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Authors' contributions RLL performed the majority of the experiments and contributed to the writing of the manuscript. JW carried out the experiments shown in Fig. 4b and confirmed other results. MRG conceived the study and prepared the final version of the manuscript. All authors read and approved the final manuscript.

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