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Review

Molecular mechanisms of *N*-acetylcysteine actions

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Abstract. Oxidative stress generated by an imbalance between reactive oxygen species (ROS) and antioxidants contributes to the pathogenesis of arthritis, cancer, cardiovascular, liver and respiratory diseases. Proinflammatory cytokines and growth factors stimulate ROS production as signaling mediators. Antioxidants such as *N*-acetylcysteine (NAC) have been used as tools for investigating the role of ROS in numerous biological and pathological processes. NAC inhibits activation of c-Jun N-terminal kinase, p38 MAP kinase and redox-sensitive activating protein-1 and nuclear factor kappa B transcription factor activities regulating expression of numerous genes. NAC can also prevent apoptosis and promote cell survival by activating extracellular signal-regulated kinase pathway, a concept useful for treating certain degenerative diseases. NAC directly modifies the activity of several proteins by its reducing activity. Despite its non-specificity, ability to modify DNA and multiple molecular modes of action, NAC has therapeutic value for reducing endothelial dysfunction, inflammation, fibrosis, invasion, cartilage erosion, acetaminophen detoxification and transplant prolongation.

Key words. Free radicals; oxidative stress; antioxidants; *N*-acetylcysteine; cell survival; signal transduction.

Introduction

Reactive oxygen species (ROS) are produced primarily by the mitochondria in cells as a by-product of normal metabolism during conversion of molecular oxygen (O_2) to water (H_2O). These include superoxide radical ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). Transfer of an electron to oxygen by cytochrome c oxidase and flavin enzymes results in production of $O_2^{-}\bullet$. The enzyme superoxide dismutase (SOD) converts it into H_2O_2 and O_2. Besides normal cells, phagocytes combat microorganisms with oxidative burst of ROS. Peroxisomes produce H_2O_2 during fatty acid degradation. H_2O_2 is mostly degraded into water by catalase, but some may also escape into the cell [1]. Cells have several antioxidant defense mechanisms. These include vitamins C and E, and enzymes such as SOD, catalase and glutathione peroxidase [2]. Oxidative stress is generated when there is an imbalance between oxidants and antioxidants. ROS can modify or damage macromolecules in cells including oxidation and peroxidation of DNA [3], proteins and lipids that can be detected either indirectly or visualized directly by histochemical means [4]. ROS at low concentrations can also serve as mediators for a variety of signal transduction pathways and ultimately gene expression [5, 6]. Both oxidants and antioxidants have a profound impact on the expression of genes [7–9]. One of the antioxidant defenses in cells is endogenous thiols (sulfhydryl-containing compounds) such as glutathione and thioredoxin [10].

N-acetylcysteine (NAC) is a thiol, a mucolytic agent and a precursor of L-cysteine and reduced glutathione. NAC is

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a source of sulfhydryl groups in cells and scavenger of free radicals as it interacts with ROS such as OH• and H_2O_2 [11]. Uses of NAC in different diseases including cancer, cardiovascular diseases, human immunodeficiency virus (HIV) infections, acetaminophen-induced liver toxicity and metal toxicity have been reviewed previously [12, 13]. The current review focuses on the recently studied effects of NAC in a variety of biological and pathological processes with particular emphasis on the molecular mechanisms of its action.

Molecular targets of NAC in cardiovascular and respiratory systems

Role of NAC in elucidating endothelial function

Oxidative stress is a major contributor of cardiovascular diseases, e. g. atherosclerosis, and although controversial, certain epidemiological studies suggest a beneficial role of antioxidants for these diseases [14]. One of the earlier steps in the initiation and development of atherosclerosis is the generation of dysfunctional endothelium generally believed to be a result of oxidative stress. Vascular endothelial dysfunction is characterized by an altered vasorelaxation and increased adhesiveness due to increased cell surface expression of vascular adhesion molecules such as VCAM-1, ICAM-1 and E-selectin that serve as receptors for circulating leukocytes in the arteries. Vasorelaxation properties of a vessel are determined by a balance between the generation of superoxide and nitric oxide (NO) in endothelium. Since superoxide can react with the vasorelaxant NO to produce peroxynitrite (a strong oxidant), increased production of superoxide can inhibit the vasorelaxation properties of a vessel by decreasing the level of available NO (fig. 1). Thus, NAC could improve the vasorelaxant properties of a vessel by suppressing the endogenous levels of ROS, including superoxide, and thereby increasing the bioavailability of NO. Cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL-1), among others, stimulate the expression of VCAM-1, ICAM-1 and E-selectin in endothelial cells. Antioxidants, NAC and pyrrolidine dithiocarbamate (PDTC), suppressed the cytokine-stimulated expression of VCAM-1 (90%) by inhibiting the binding of nuclear factor kappa B (NF-κB) to the κB motif of VCAM-1 promoter. Under the same conditions antioxidants did not affect the expression of ICAM-1. Furthermore, PDTC was also shown to inhibit VCAM-1-medi-

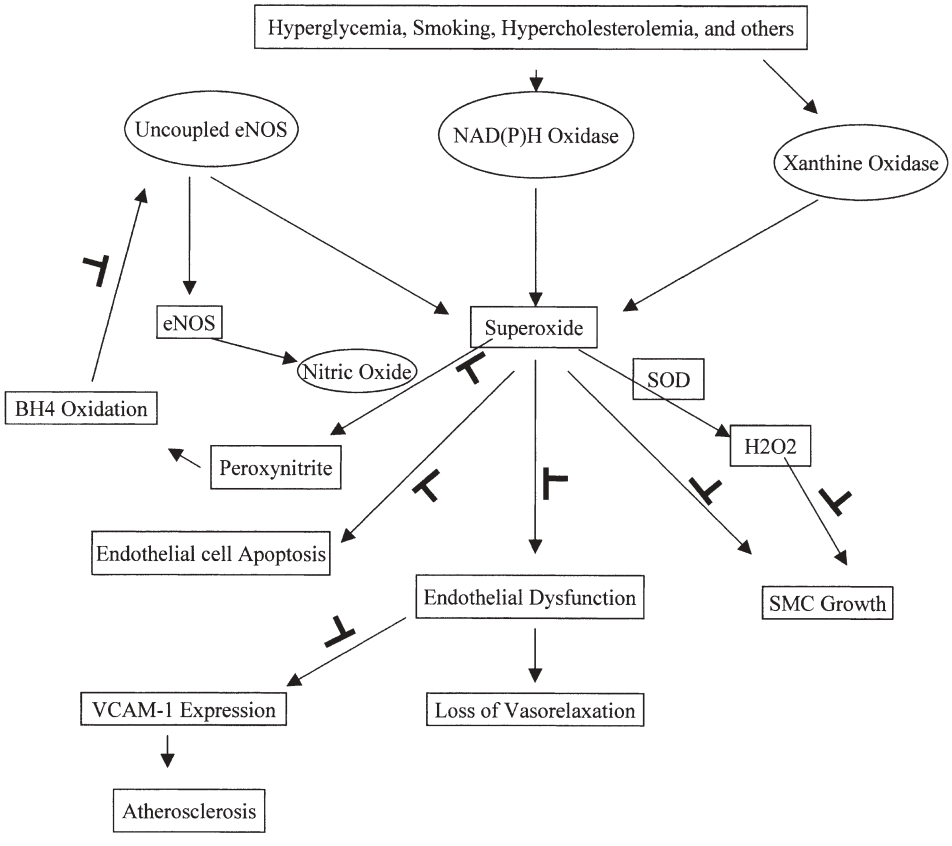

Figure 1. Extracellular mediators of cardiovascular functions and roles of NAC. The activation steps by various risk factors are indicated by arrows, and inhibition by NAC is depicted by the symbol ⊥. Abbreviations are e-NOS, endothelial nitric oxide synthase; BH4, tetrahydrobiopterin; SOD, superoxide dismutase; SMC, smooth muscle cells; VCAM-1, vascular cell adhesion molecule-1.

ated cellular adhesion, suggesting that the adhesiveness of endothelium can be inhibited by antioxidants [15]. Studies from another laboratory also suggested that several radical-generating systems are involved in the induction of VCAM-1 by TNF and that NAC and PDTC can inhibit the adhesion of monocytic U937 cells to TNF-treated human umbilical vein endothelial cells (HUVECs) [16]. In another report, the IL-1-stimulated expression of both VCAM-1 and E-selectin was shown to be inhibited by NAC. However, NAC inhibited the expression of these genes through different mechanisms. As observed earlier, NAC inhibited the expression of VCAM-1 by inhibiting the binding of NF-κB to the VCAM-1 κB motif. Under the same conditions, NAC did not inhibit the binding of NF-κB to the E-selectin κB motif, suggesting that NAC inhibits E-selectin expression through a different mechanism [17]. Since NF-κB has been shown to be necessary for the cytokine-stimulation of expression of VCAM-1, ICAM-1 and E-selectin, the differential effect of NAC on the binding of NF-κB to the κB motif of different adhesion molecule genes suggests that multiple NF-κB complexes may be involved in regulating the expression of these vascular adhesion molecules and that the activity of NF-κB that regulates the expression of VCAM-1 is redox sensitive. In pulmonary artery endothelial cells, based upon TNF-induced ROS production and their inhibition by NAC, ROS involvement in NF-κB activation and ICAM-1 and E-selectin expression was suggested [18, 19]. It is possible that adhesion molecule genes in HUVEC and pulmonary artery endothelial cells have a differential redox sensitivity. In another study with HUVEC, however, NAC had no impact on cytokine-induced E-selectin expression, while several protein thiol-modifying agents inhibited it [20]. In a study investigating the mechanisms of diabetes-associated atherosclerosis, advanced glycation end products were found to augment NF-κB activity and VCAM-1 expression that were inhibited by NAC, affirming the role of ROS in the development of diabetic vasculopathy [21]. NAC specifically prevented TNF-activated inhibitor of NF-κB (Iκ-B) kinase-mediated Iκ-Bα phosphorylation and degradation without affecting β and ζ Iκ-Bs [22]. This agent was utilized to demonstrate that preconditioning of endothelial cells against ischemia via induction of adhesion molecules (ICAM-1, E-selectin), NF-κB activation and subsequent protection was oxidative stress dependent [23]. NAC and catalase also abolished the endothelial induction of ICAM-1 (70%) and VCAM-1 (100%), respectively, by cyclic strain and by oscillatory shear stress, suggesting redox sensitivity of the signal transduction mechanisms [24, 25]. The role of the extracellular signal-related kinase (ERK) pathway and ROS in the signaling of cyclic strain-induced early growth response-1 gene expression was demonstrated by their suppression with NAC [26]. This agent also completely blocked IL-4-induced oxidative stress and downstream activation of Sp1 transcription factor and VCAM-1 expression [27]. By increasing glutathione levels via NAC treatment, many harmful effects of TNF-α related to endothelial dysfunction could be partially overcome [28]. NAC reduced GTPase Rac1-induced superoxide production and blocked cytoskeletal reorganization in endothelial cells [29]. Although under TNF-induced proinflammatory conditions NAC blocks ICAM-1 and VCAM-1 expression, under noninflammatory conditions (without TNF), this agent increased expression of these genes in HUVEC and human aortic cells in some studies [30, 31].

Pretreatment of HUVEC with NAC decreased heme-induced oxidative stress and ICAM-1 expression by 37% [32]. Leptin, which is associated with the human obesity and atherosclerosis, induced oxidative stress mediators such as ROS, JNK and NF-κB activation, and NAC completely inhibited these events, thereby demonstrating redox-sensitive signaling of hyperleptinemia [33]. Induction of the major endothelial mitogen, vascular endothelial growth factor (VEGF) by H_2O_2 (a prominent ROS and a second messenger) was suppressed by NAC in rat heart endothelial cells [34]. The decrease in cardiovascular mortality by antioxidants in epidemiological studies may be partly due to their (nordihydroguaiaretic acid and NAC) ability to enhance three times the endothelial nitric oxide synthase (eNOS) expression and NO bioactivity, a potent vasodilator, which is reduced in hypertension and atherosclerosis [35]. NAC-mediated increase in glutathione levels resulted in the induction of inducible NOS (iNOS) gene transcription and messenger RNA (mRNA) expression [36]. NAC also protects endothelium from cigarette smoke-induced injury and cell death, an important risk factor for vascular diseases [37, 38]. By increasing cellular glutathione, NAC was able to attenuate TNF-α-induced p38 mitogen-activated protein kinase (MAPK) activity in human pulmonary vascular endothelial cells, suggesting redox regulation of p38 MAPK pathway and protective role of antioxidants in lung injury [39]. While investigating the mechanisms of hyperglycemia-induced proatherogenic changes in endothelial cells, NAC was shown to block glucose-stimulated ERK5 activity [40]. Induction of heme oxygenase (HO-1) by TNF-α and IL-1 in endothelial cells is also partly ROS dependent that can be inhibited by NAC [41]. Peroxynitrite generated by interaction of NO and superoxide anion leads to the formation of hydroxyl radical, induction of HO-1 and endothelial cell apoptosis. NAC completely abolished HO-1 increase and activity [42]. This protein may have a protective role against apoptosis. Oscillatory shear- and laminar shear stress-induced HO-1 upregulation could be inhibited by NAC in endothelial cells; however, the former induced a sustained prooxidant response while the latter also activated antioxidant defenses [43]. Shear flow-induced *c-Fos* gene expression was mediated by

ROS as this response was inhibited (50%) by NAC [44]. NAC inhibited shear-induced tyrosine phosphorylation in bovine endothelial cells, which is mediated by Rac-1-dependent ROS production [45]. Overall, these studies suggest that NAC could improve endothelial function and may attenuate vascular inflammatory disease (atherosclerosis) by antagonizing the effects of intracellular ROS generation, by increasing the bioavailability of nitric oxide and by reducing leukocyte adhesion to the endothelium (fig. 1). Further NAC may also be beneficial for treating obesity-related endothelial dysfunction and for attenuating ischemia-reperfusion injury that remains to be studied in patients with these disorders.

Impact of NAC on the functions of vascular smooth muscle cells (VSMCs)

A major problem in cardiovascular diseases (atherosclerosis and restenosis) is excessive proliferation of VSMCs. NAC partially inhibited the synergistic ox-LDL (a prooxidant) and urotensin (a potent vasoconstrictor)-stimulated proliferation of VSMCs, a useful concept for reducing rapidly progressing atherosclerosis in patients with hypertension and hypercholesterolemia [46]. The role of ROS via p38 MAPK pathway regulation in differentiation phenotype of VSMCs was demonstrated through 40% inhibition by NAC and catalase [47]. Additionally, NAC inhibited serum-, PDGF- and thrombin (inducers of VSMC DNA synthesis and proliferation)-stimulated ERK2, JNK1 and p38 MAPK activation as well as expression of the c-*Fos* (70%), c-*Jun* (50%) and *JunB* (70%) genes, suggesting redox-sensitive mechanisms for these events [48]. PDGF signal transduction in VSMCs was modulated by H_2O_2 production that could be inhibited by NAC [49]. Subsequently, constitutively active Ha-Ras was shown to induce superoxide and mitogenesis of NIH 3T3 fibroblasts that could be blocked by NAC [50]. NAC completely inhibited angiotensin II-stimulated downregulation of angiotensin II type I receptor by ROS production and by stabilizing its destabilized mRNA [51]. It also blocked serotonin-stimulated superoxide production and ERK-MAPK phosphorylation in VSMCs; however, the two pathways may be independent of each other [52]. Redox sensitivity of lactosylceramide signaling and related proliferation was demonstrated by inhibition of p21 ras GTP loading, p44 MAPK activation (70%) and c-*Fos* (100%) expression [53]. In VSMCs, NAC inhibited cyclooxygenase-2 induction by benzo (a) pyrene, an atherogenic ingredient of cigarette smoke. This suggested glutathione dependence of its gene expression [54]. Thus, NAC has the ability to interfere with the signaling pathways of different stimuli (native and ox-LDL, angiotensin II, growth factors and advanced glycation end products) implicated in VSMC proliferation and migration. Indeed, NAC reduced thickening of neointima by 39% in rabbit aorta injured by balloon, an animal model of restenosis [55]. It remains to be studied whether NAC will reduce VSMC activation in patients with restenosis, hypertension and diabetes.

Role of NAC in atherosclerotic plaque stability

ROS such as superoxide, NO and H_2O_2 generated by macrophage foam cells can modulate the activities of matrix-degrading proteases, matrix metalloproteinases (MMPs) and contribute to the instability of vulnerable atherosclerotic plaque [56]. ox-LDL has been shown to activate AP-1 and NF-κB transcription factors, induce MMPs (MMP-9), downregulate their inhibitor, TIMP-1, and promote macrophage-mediated matrix disruption in the rupture-prone atheroslecrotic plaques [57]. NAC inhibited the homocysteine (a risk factor in cardiovascular diseases)-enhanced expression of an ox-LDL receptor, lox-1 in endothelium [58]. ox-LDL also induces MMP-1 and MT1-MMP in endothelial cells and VSMCs [59, 60], cell types partly contributing to plaque rupture. MMP-9 (gelatinase B) activity and expression in lipid-laden macrophage-derived foam cells as well as in rabbit aortic explants could be inhibited up to 60% by 10 mM of the antioxidant, NAC [61]. Thus, ROS and redox status seem to regulate expression and activities of MMPs, and antioxidants could potentially stabilize vulnerable plaques. This remains to be confirmed by in vivo studies. Our unpublished results show that NAC and other antioxidants downregulate the expression of tissue inhibitor of metalloproteinase-3 (TIMP-3), a major MMP inhibitor and an inducer of apoptosis in VSMCs. Thus antioxidants could potentially further destabilize the plaques and increase restenoic proliferation of VSMCs. It also remains to be tested whether exogenous NAC or the recently described protective endogenous antioxidants such as heme oxygenase-1 [62] and metallothionein [63] are superior for protecting vascular system.

While in vitro systems are convenient for studying detailed molecular mechanisms, effects of antioxidants need to be studied in animal models of the disease or human clinical trials. NAC treatment in rabbits reduced angioplasty-induced vascular inflammation, thrombus formation and laminal damage [64]. By using hypertensive rats, NAC administration was shown to be partially protective against peroxynitrite-induced aortic vascular dysfunction related to hypertension [65]. NAC treatment gave some protection in rats subjected to ischemic and reperfusion injury in part by inhibiting adhesion molecules [66]. In cardiac myocytes, reoxygenation-induced ROS production and phosphorylation of JNK was inhibited by NAC, which may be another target for conferring protection [67]. The mechanism of protection against the ischemia-reperfusion-induced injury of heart and lung by NAC and thioredoxin, may be different [68]. Patients

containing elevated levels of remnant-like lipoprotein (RLP) treated with α-tocopherol (another antioxidant) displayed decreased adhesion molecules. NAC inhibited RLP-increased adhesion molecules by 50–70% in cultured endothelial cells and restored impaired endothelium-dependent vasorelaxation [69]. A clinical trial showed that oral dose of 1.2 mg of NAC per day increased glutathione (GSH) and decreased plasma VCAM-1 levels in non-insulin-dependent diabetic patients [70]. In a group of patients, NAC supplementation significantly improved coronary and peripheral vasodilation by enhancing the effects of NO [71].

NAC in respiratory system

In the mouse model of lipopolysaccharide inhalation and subsequent lung inflammation, antiinflammatory and antioxidant activity of NAC was much lower compared to dexamethasone, and the two agents had different mechanisms of action [72]. In another study, pretreatment of mice with NAC prior to endotoxin injection resulted in reduced NF-κB activation and neutrophilic alveolitis [73]. In human bronchial epithelial and lymphoma cells, NAC inhibited silica- or TNF-induced NF-κB activation (up to 80%) and interleukin-8 increase that was demonstrated to be ROS dependent [74, 75]. In human bronchial epithelial cells, TNF-α-induced activation of p38 MAPK and RANTES (a chemokine) production was dependent upon cellular redox status regulated by glutathione because it was attenuated by NAC [76]. TNF-α contributes to the loss of lung allograft, and NAC was shown to attenuate TNF-α mRNA expression and secretion in macrophages from human lung transplant recipients and may be beneficial against transplant rejection [77]. NAC administration in rats protected against cigarette smoke-induced histopathological changes, including bronchial inflammation, emphysema and precancerous lesions [78]. Transforming growth factor β (TGF-β) causes pulmonary fibrosis and inhibits growth of artery endothelial cells that can be reversed by increasing glutathione levels with NAC, cysteine and cystine [79]. However, subsequent studies showed that at low doses (0.1–1 mM) NAC works as regulator of redox state, but at elevated doses (10 mM), it can directly alter the structure of TGF-β [80]. We have evidence that TGF-β signaling is redox sensitive and can be inhibited by NAC in different cell types, although the precise mechanism is not known [W. Q. Li et al, unpublished results]. Inhibition of TGF-β signaling or its direct modification by NAC can be interesting in several diseases where an excessive amount of this factor is involved in pathogenesis. NAC may be useful for reducing lung inflammation, transplant rejection and pulmonary fibrosis. Thus, NAC has been used for blocking several pathways of major interest in cardiovascular and respiratory systems (fig. 2).

***N*-acetylcysteine in musculoskeletal system and arthritis**

In a mouse model of lupus (an autoimmune disease), NAC treatment resulted in significant suppression of anti-DNA antibodies and a modest increase in survival [81]. TNF-α is a major stimulus for inflammatory arthritis such as rheumatoid arthritis (RA) where it instigates synovial inflammation, hyperplasia and cartilage degeneration, the main symptoms of the disease. This cytokine and basic fibroblast growth factor were shown to induce c-*Fos* gene expression in cartilage cells (chondrocytes) by NADPH-generated ROS as signaling mediators that was inhibited by NAC [82]. Similarly, based upon ROS production, stimulation by H_2O_2 and inhibition by NAC, it was demonstrated that ROS (H_2O_2 and to a lesser extent, NO) constitute second messengers for IL-1 and TNF-induced JNK activity and c-*Jun* gene expression [83]. Since c-Fos and c-Jun are the major components of AP-1 transcription factor, these studies demonstrated its sensitivity to reduction-oxidation and revealed novel targets for antioxidant therapy in diseases such as arthritis and cancer. TNF-α induces cartilage destructive MMPs by the JNK signaling pathway, which culminates on AP-1 transcription factor binding sites found in several MMP gene promoters. Indeed, by neutralizing ROS, NAC was shown to inhibit IL-1β-induced c-*Fos* (by 40%) and collagenase (by 60%) gene expression in chondrocytes [84]. We also showed that NAC could effectively (36–100%) inhibit TNF-α-induced MMP-3 gene expression in synovial fibroblasts [85]. Fragments of the extracellular matrix protein, fibronectin, penetrate and cause proteoglycan depletion by mediation of inflammatory cytokines and ROS, enhancing cartilage damage by MMPs such as MMP-3. NAC was found to totally suppress cartilage explant (where chondrocytes are found in their native matrix) proteoglycan depletion and MMP-3 activities stimulated by these fragments as well as by ROS (H_2O_2 and superoxide anion), IL-1α and TNF-α [86]. In related studies, NAC and glutathione were shown to restore proteoglycan levels by suppressing the effects of catabolic cytokines and promote cartilage repair in the ex vivo cartilage explant model [87, 88]. NAC was able to partially reverse the inhibition of proteoglycan synthesis and chondrocyte death induced by 4-day exposure of human cartilage explants to blood, a source of ROS in this in vitro model of joint bleeding-stimulated damage [89]. However, impact of NAC was not studied in a canine model of joint bleeding [90].

ROS have been implicated in the aging of cartilage and osteoarthritis (OA). Several types of cells in joints can produce ROS, including phagocytes and chondrocytes [91]. ROS production has been observed in articular chondrocytes [92]. Superoxide anions could reduce polymerization of the joint protective, hyaluronic acid (HA) in

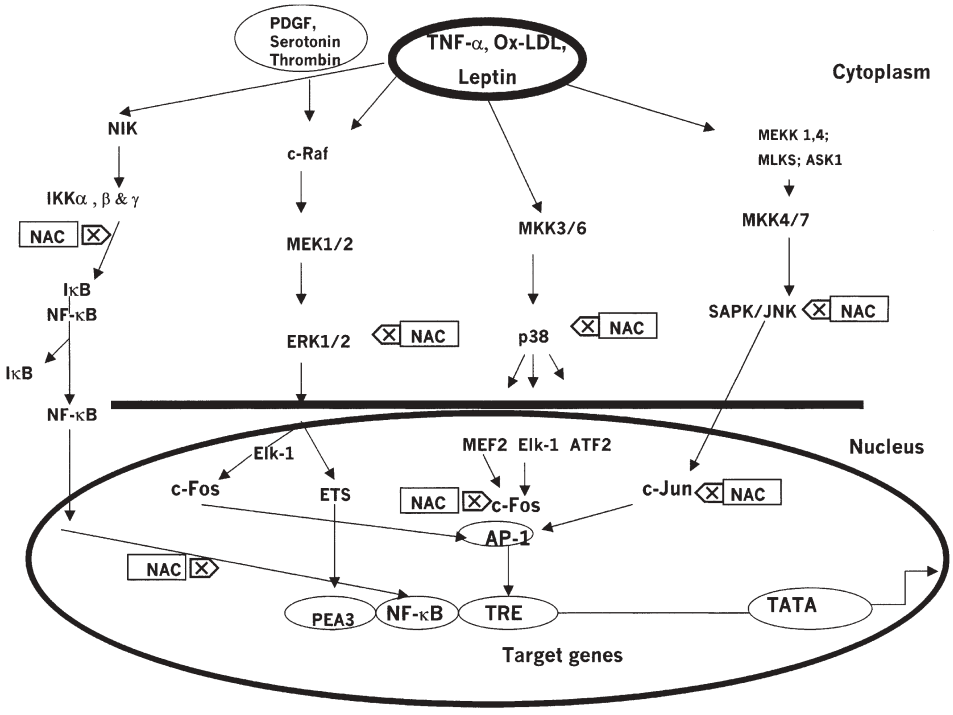

Figure 2. Main signaling pathway targets inhibited by NAC. The activation steps are indicated by arrows, and inhibition by NAC is depicted by ⊠ or ⊠. Abbreviations are PDGF, platelet-derived growth factor; TNF-α, tumor necrosis factor α, Ox-LDL, oxidized low-density lipoprotein; NF-κB, nuclear factor kappa B; NIC, NF-κB-induced kinase; IKK, IκB kinase; IκB, inhibitor of NF-κB; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; ELK-1, ets domain protein; MEF2, myocyte enhancer factor 2; ATF2, activating transcription factor 2; TRE, tumor promoter responsive element; AP-1, activator protein-1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase.

the synovial fluid, a process reversed by the antioxidant enzyme SOD [93]. NAC and cysteine, however, gave limited protection against HA depolymerization [94]. HA is currently a popular though controversial remedy in osteoarthritis. It has the ability to scavenge IL-1-induced superoxide anion and may give protection against cartilage degradation [95]. H_2O_2 causes fragmentation and chemical modification of various components of aggrecan, the major proteoglycan of cartilage [96, 97]. ROS may also modify collagen synthesis and structure, and can degrade HA [98]. Antioxidants such as catalase, mannitol and thiourea decreased aggrecan damage. ROS (H_2O_2) also cause lipid peroxidation in cartilage that may contribute to its aging and osteoarthritic degeneration. The chain-breaking antioxidant, vitamin E, but not antioxidant enzymes reduced matrix release, suggesting the role of lipid peroxidation in matrix collagen oxidation, degradation and release [99]. Lipid peroxides are potent inducers of cartilage-damaging MMPs in rheumatoid synovial fibroblasts that are known to invade cartilage [100]. NAC (although not tested) may be beneficial against such lipid peroxidation-mediated damage. In view of the above observations, antioxidant therapy in arthritis may be relevant, as the risk of RA is increased in the patients with low levels of antioxidants such as α-tocopherol and β carotene [101]. Further, T cells from patients with RA are hyporesponsive to mitogenic agents due to deficiency in glutathione levels and altered redox state that could be restored with NAC [102]. The course of OA (a degenerative arthritis associated with injuries and aging) can possibly be altered by dietary antioxidants such as vitamin C [103, 104].

In IL-β-stimulated osteoblasts, NAC could inhibit NF-κB translocation and cyclooxygenase-2 (COX-2) expression, a major target of the antiinflammatory drugs [105]. IL-6, an elevated cytokine in RA, induced ROS and rheumatoid synoviocyte proliferation which could be inhibited by NAC [106]. In human rheumatoid fibroblasts, NAC inhibited TNF-induced ROS-mediated nuclear translocation of NF-κB, synthesis of IL-6 and IL-8, ICAM-1, monocyte chemoattractant protein-1 and collagenase; the proteins contributing to proliferation, adhesion to cartilage and invasion of this tissue [107, 108]. NAC decreased NF-κB-mediated proliferation of these synovial cells [109]. In rabbit synovial fibroblasts, integrin activated Rac-1, ROS production, NF-κB activation and IL-1α induction, leading to increased collagenase-1 gene expression; NAC blocked ROS and IL-1α production [110]. IL-18-induced VCAM-1 expression (implicated in synovial adhesion and invasion of cartilage) was inhibited by

50–60% in human rheumatoid fibroblasts [111]. In agreement with these in vitro studies, NAC was shown to inhibit collagen-induced arthritis in mice by inhibiting inflammatory cytokines and NF-κB activity [112, 113]. In a human exercise model of acute muscle injury, NAC (10 mg/Kg body weight for 7 day post-injury) transiently increased oxidative stress and tissue damage, although effects of chronic use of this supplement are not known [114]. These studies suggest that the beneficial effects of NAC in individuals may depend on their health condition. Overall, NAC may be useful for treating joint inflammation, synovial invasion of cartilage and its degradation in arthritis, but it may have adverse effects in healthy individuals performing eccentric exercise.

N-acetylcysteine in cancer

NAC has been shown to prevent in vivo carcinogenesis, invasion and metastasis of endothelial cells in part by inhibiting conversion of preprogelatinase (MMP-2) to active progelatinase form and its enzyme activity. Furthermore, NAC prevented angiogenesis, a major mechanism for tumor growth, without inducing apoptosis of endothelial cells [115, 116]. NAC and ascorbic acid combination was more effective relative to NAC alone in reducing urethane-induced lung tumor burden and size in mice [117]. Cigarette smoke-induced apoptosis of bronchial epithelium as a defense mechanism against its genotoxicity as well as DNA adduct formation was inhibited by NAC [118, 119]. The role of NAC as an effective chemopreventive agent in cigarette-smoke-induced cancer has been comprehensively discussed in an excellent recent review analyzing several carcinogenesis-related molecular mechanisms [120]. In contrast with these suggestions, no benefit of NAC supplementation (600 mg/day) was observed in a 2-year clinical trial with patients with head, neck or lung cancer [121]. NAC inhibited tumor promoter-induced invasion of human bladder cancer cells by reducing MMP-9 production and activity [122]. Along with hydrostatic pressure, NAC was shown to eliminate metastasis of tumor cells [123]. NAC inhibited cell survival-associated constitutively active NF-κB and growth of malignant melanoma cells [124]. In peripheral blood mononuclear cells of patients with advanced cancer, NAC increased CD25- and CD95-expressing cells thus restoring their normal function [125]. Compared with a placebo group of patients, NAC (800 mg/day)-treated patients with colonic polyps had significantly reduced proliferative index, a predictive biomarker for increased colon cancer [126]. NAC is able to reduce Copper (II) to Cu (I), stimulate Cu (I)-dependent H_2O_2 production and subsequent oxidative damage to DNA, and thereby is suggested to have both carcinogenic and anticarcinogenic activities. NAC slightly enhanced DNA oxidative damage generated by an estrogen (4-hydroxyesterdiol) and Cu (II) [127, 128]. A fivefold reduction of ROS by NAC in colon carcinoma cells increases the proapoptotic, *bax* gene expression and effectiveness of chemotherapeutic agent, 5 fluorouracil [129]. NAC and penicillamine were shown to induce apoptosis in several transformed cell lines but not in normal cells. NAC increased p53 (a tumor suppressor gene) RNA translation in normal and transformed cells, which may be mediating apoptosis, a major therapeutic target in cancer. Further, transformed cells are selectively sensitive to NAC [130]. Some of the beneficial effects of NAC may be due to induction of cyclin-dependent kinase inhibitors and its ability to arrest cell cycle at the G1 stage; these properties are independent of increase in glutathione [131, 132]. Aside from these limited studies showing anticancer activities of NAC, most investigations, however, showed that NAC inhibited cancer cell apoptosis under a variety of conditions. This property of NAC has been used to block neurotoxicity and apoptosis induced as a side effect of anticancer agents such as cisplatin [133]. A recent study showed that dietary NAC reduced 8-hydroxyguanine (a biomarker of oxidative DNA damage) by 50% and the mutagenic potential of oxidized DNA in mouse but induced additional, potentially deleterious structural changes affecting fidelity of DNA synthesis [134]. Thus, NAC appears to have some benefits for certain cancers as an adjuvant therapy but may also have side effects.

Promotion of cell survival and antiapoptotic activities of NAC

Oxidative stress causes programmed cell death or apoptosis in several pathological processes, including aging, inflammation, carcinogenesis, neurodegeneration and arthritis (reviewed in [135]). From studies of various cell types, it is becoming increasingly clear that NAC has growth-promoting activities. In one study, NAC further increased concanavalin A-induced mitogenesis and simultaneously reduced apoptosis of B lymphocytes [136]. Additionally, upon detachment and maintenance in suspension culture (anoikis), endothelial cells produce elevated levels of ROS, caspase and JNK activities and undergo apoptosis, which is blunted by NAC, increasing viability by twofold. The pathophysiological significance of this in vitro observation is not known [137]. NAC and dithiothreitol (DTT) were shown to block apoptosis of endothelial cells by lipopolysaccharide [138]. ox-LDL-induced superoxide production and apoptosis of HUVEC were blunted by NAC [139]. In contrast with endothelial cells, NAC and PDTC induced apoptosis and reduced viability of rat and human VSMCs [140]. NAC was found to maintain VSMCs in quiescent state, and its removal led to their reentry in the cell cycle, a useful approach for

synchronization of these cells [141]. During investigation of the mechanisms of hyper-homocysteinemia-associated atherosclerosis, NAC suppressed homocysteine-stimulated collagen synthesis and proliferation of VSMCs [142]. Such selective impact of NAC can be useful for blocking proliferation of VSMCs in atherosclerotic and restenoic lesions. Sodium butyrate-induced apoptosis in Chinese hamster ovary cells was inhibited by NAC treatment [143]. Copper-induced ROS and apoptosis were inhibited by NAC in a B cell line [144]. While studying the effect of an antioxidant, Ebselen, on hepatoma cell death, this agent was found to induce apoptosis by depleting thiols, and NAC rescued HepG (2) cells from apoptosis [145].

In the absence of exogenous nerve growth factor (NGF), neurons undergo apoptosis that can be prevented by NAC, possibly via its reducing rather than its antioxidant or glutathione-enhancing activity [146]. This happens in part by activation of the Ras-ERK pathway, inhibition of the JNK pathway and induction of early response genes, *c-Fos* and *c-Jun* [147]. Semaphorins are axon guidance molecules and have chemorepulsive activity during developmental axonal navigation to correct destinations. Neuronal apoptosis induced by a semaphorin-3A peptide could be inhibited by NAC and VEGF [148]. NAC also prevented TNF- and thrombin-induced neuronal cell death [149, 150]. It has been suggested that ERK-MAPKs can activate ribosomal S6 kinases (RSKs), which phosphorylate BAD at serine 112 and inhibit apoptosis. RSKs can also phosphorylate CREB at serine 133, which promotes cell survival [151]. Arabinoside-induced cerebral neuron apoptosis and resulting neurotoxicity were inhibited in vitro by ROS inhibition and DNA damage by NAC [152]. Apoptosis of F-MEL cells induced upon serum deprivation can be prevented by NAC [153]. NAC may promote survival of neurons and possibly other cell types by such a mechanism. It is possible that at low concentrations, NAC promotes cell growth and at elevated doses induces apoptosis.

Chondrocyte apoptosis has been considered as a major contributor of cartilage degradation in RA and OA patients [154–156], although a recent study suggests that apoptosis is not a widespread phenomenon in arthritis [157]. Chondrocytes, the only cell type in cartilage, are very sensitive to ROS such as H_2O_2 and OH• in culture but can survive in the presence of endogenous or exogenous antioxidants such as cysteine, dithioerythritol and catalase [158]. Furthermore, thiols such as cysteine, cystine and glutathione can promote their survival [159]. We have recently found one of the possible mechanisms for these observations and demonstrated that addition of NAC, L-cysteine, glutathione and PDTC to mitogen-free human and bovine articular chondrocyte cultures rapidly activates the ERK signaling pathway, a MAPK cascade usually associated with the growth-factor-dependent cell survival and proliferation [160, 161]. Downstream consequences of ERK activation in chondrocytes are not known, but the ability of NAC to inhibit the JNK pathway may lead to prosurvival activities (Bcl2 and Akt activation?). Interestingly, NAC at very low concentrations was found to transiently induce early-response, c-fos mRNA expression, which may be a consequence of ERK pathway activation [162]. The two pathways are known to work in opposition; ERK being growth promoting and JNK being proapoptotic [162]. In other systems, AP-1 activity was induced by NAC via sequential activation of the ERK-MAPK pathway and Elk-1 transcription factor, which binds to the serum response element in the *c-Fos* promoter and induces its gene expression [163, 164]. This activity of NAC may lead to cell growth and differentiation. The in vivo implications of this interesting observation need to be tested in animal models of arthritis and by studying the impact of dietary supplementation of antioxidants on human arthritis. Some of the observed beneficial effects of NAC in collagen-induced mouse arthritis models and repair activities in cartilage explant cultures may be partly due to blockade of the JNK pathway, prevention of apoptosis and stimulation of such survival pathways (fig. 3). Interestingly, inhibition of glutathione blocks and its increase by NAC restores chondrocyte differentiation in rabbit limb bud micromass [165]. Overall, these results suggest that NAC may have beneficial effects through multiple mechanisms, depending on the cell type. While some of the beneficial effects are mediated through increasing the survival of cells, e.g. endothelial, chondrocytes and neurons, others are mediated through decreasing the survival, e.g. smooth muscle cells.

N-acetylcysteine in liver physiology and diseases

Oxidative stress generated by various conditions activates hepatic stellate cells (HSCs) and constitutes a possible link between chronic liver damage and hepatic fibrosis (production of excessive fibrous tissue), ultimately leading to cirrhosis. NAC administration in rats reduced dimethylnitrosamine (a profibrotic agent)-induced and fibrosis-associated fibronectin deposit [166]. At the molecular level, NAC arrested HSCs at the G1 phase of the cell cycle by modifying the redox status of cysteines in Raf-1, MEK and ERK signaling proteins, resulting in sustained activation of ERK-MAPK, induction of the cell cycle inhibitor p21Cip1 and increased Sp1 phosphorylation. These actions of NAC were related to its reducing activity [167]. Coadministration of NAC with toxic cadmium in rats diminished lipid peroxidation and gave protection against hepatic toxicity, suggesting the role of oxidative stress in the toxicity [168]. Administration of NAC within 10–18 h in patients with acetaminophen overdose and alcoholism prevents liver damage and sig-

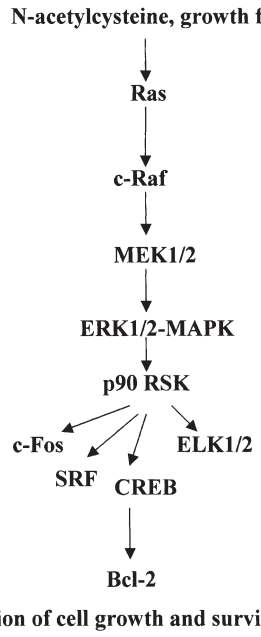

Figure 3. A model proposing the mechanisms of NAC-stimulated cell survival. NAC activates the extracellular signal-regulated kinase, mitogen-activated protein kinase (ERK-MAPK) pathway, which in turn can activate multiple transcription factors and genes implicated in cell growth and survival. ELK-1 and serum response factor (SRF) can also bind with the promoter of *c-Fos* gene and increase its transcription. RSKs (ribosomal S6 kinase) can phosphorylate BAD at serine 112 and suppress BAD-mediated apoptosis. RSKs also phosphorylate cyclic AMP response element binding protein (CREB) at serine 133 and promote cell survival. (Based on [146, 151, 160–162]). Conversely, blocking ERK-MAPK in chondrocytes and possibly other cell types leads to apoptosis.

nificantly reduces mortality [169, 170]. The possible mechanisms of antitoxicity include improved liver blood flow, glutathione replenishment and free radical scavenging [171]. Cocaine-induced mitochondrial damage in hepatocytes was partially reduced by NAC [172]. Liver injury by oxidative stress during reperfusion of liver transplantation can be significantly reduced by NAC treatment through downregulation of α-glutathione S-transferase and circulating adhesion molecules, ICAM-1, VCAM-1 in the donor liver [173]. NAC treatment of ischemia and reperfusion-injured rat livers blocked NF-κB activity and iNOS expression [174]. Hepatocyte apoptosis induced by transplantation-related cold storage of rat liver could be diminished by NAC treatment, and its addition before rewarming may be beneficial for successful transplantation [175]. Hepatitis C virus nonstructural protein 5A or hepatitis B virus X protein generate oxidative stress in the liver and activate NF-κB and STAT3 transcription factors that can be eliminated by NAC [176–177]. NAC and related agents such as *S,N*-diacetylcysteine monoethyl ester could improve the status of protective sulfhydryl compounds such as glutathione in rat liver [178]. Glutathione levels depleted by acetaminophen overdose in HIV-infected patients can be replenished by NAC. Thus, NAC has therapeutic potential for liver against oxidative stress, transplantation-related damage, fibrosis, hepatitis, acetaminophen overdose, chronic alcoholism and heavy metal toxicity.

Conclusions

NAC can scavenge ROS, increase glutathione levels, undergo autooxidation (and produce H_2O_2) and serve as reducing agent. Activation of NF-κB in response to a variety of signals (IL-1, TNF, H_2O_2) can be inhibited by NAC, suggesting ROS as common signaling modulators. NAC has been an extensively utilized tool for investigating redox sensitivity of biological or pathological processes. However, due to multiple activities of NAC and the possibility of direct modification of certain signals and signaling proteins, caution is warranted in interpretations. Such putative redox-sensitive mechanisms should be confirmed by additional approaches such as overexpression of antioxidant enzymes and proteins. NAC can interfere with cell adhesion, oxidative stress, smooth muscle cell proliferation by mitogens, stability of rupture-prone atherosclerotic plaques in the cardiovascular system and reduce lung inflammation, fibrosis, smoking-related changes and prolong transplants. In arthritis it can reduce inflammation, synovial invasion and cartilage damage. In cancer, this agent inhibits angiogenesis, reduces smoking-induced carcinogenesis, induces selective apoptosis of transformed cells, interferes with the cell cycle and has antiinvasive and antimetastatic effects. In certain cell types (chondrocytes, neurons) it promotes cell growth and survival. In liver, NAC diminishes oxidative stress by various agents, and gives some protection against fibrosis, viral infections and toxicity. Beneficial therapeutic effects of NAC in different diseases may be via multiple, nonspecific and sometimes contradictory (e. g. proapoptotic versus prosurvival) mechanisms. These observed effects in individual systems should be studied in relation to other systems and in models and patients as a whole. Recent observation of possible structural changes in DNA by NAC suggests caution for its clinical use and invites further research in terms of toxicity.

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