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Reactive Oxygen Species and Molecular Mechanism of Silica-Induced Lung Injury

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Silica particles are considered to be fibrogenic and carcinogenic agents, but the mechanisms of disease initiation and progression are not fully understood. This article summarizes the literature on the generation of reactive oxygen species (ROS) directly from interaction of silica with aqueous medium and from silica-stimulated cells. This article also discusses the role of ROS in silica-induced lung injury, with particular focus on the silica-induced NF-κB activation, including the molecular mechanisms of its regulation, its possible attenuation, and its relationship to silica-induced generation of cyclooxygenase II and TNF-α.

KEY WORDS: silicosis, reactive oxygen species, free radicals, NF-κB, TNF-α, cyclooxygenase II

Introduction

Epidemiological and pathological studies have established that occupational exposure to crystalline silica can lead to the development of either chronic or acute pulmonary disease.[1,2] Evidence from epidemiological and animal studies has also implicated crystalline silica as a potential carcinogen.[3,4] For example, inhalation of silica has been shown to be carcinogenic in rats.[5-9] Intrapleural administration of crystalline silica in rats leads to the induction of localized large-cell lymphomas. Epidemiological studies also showed an increased lung cancer risk in many, but not all, human subjects with silicosis.[4,9] Based on evidence obtained from both animal models and epidemiological studies, the International Agency for Research on Cancer has concluded that there is sufficient evidence that inhaled crystalline silica, in the forms of quartz or cristobalite from occupational sources, is carcinogenic to humans.[9]

In the past decade or so, various studies have demonstrated that silica-induced generation of hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$), collectively called reactive oxygen species (ROS), may cause a persistent oxidative stress in the lung and play a key role in the mechanism of silica-induced lung damage.[10,14] For example, silica particles are able to cause lipid peroxidation in vitro and in exposed workers.[15] Through free radical reactions, silica particles are also able to cause DNA strand breaks, dG hydroxylation, and thymine glycol formation.[11,16] These and related studies have contributed enormously to our understanding of the role of free radical reactions in the mechanism of fibrosis and cancer induced by silica in the past decade or so. Recently, a subdiscipline of molecular toxicology and carcinogenesis has developed. New techniques are now available to help researchers understand the mechanism of silica-induced cellular injury and the role of free radical re-

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actions in precise molecular terms. For example, what molecules act as intracellular mediators of various silica-induced responses? What are the signal transduction pathways involved? This article focuses on silica-induced free radical generation, activation of nuclear transcription factor (NF-κB) and the role of free radical reactions in the activation process, the possible attenuation of silica-induced NF-κB activation, and its relationship to silica-induced generation of cyclooxygenase II and TNF-α.

Silica-Induced ROS Generation

Silica can induce ROS generation by direct interaction with aqueous medium and/or by stimulation of cells.

Free radical generation directly from silica. During the late 1980s, electron spin resonance (ESR) studies demonstrated the generation of silicon-based free radicals ($Si^{\bullet}$, $SiO^{\bullet}$, $SiOO^{\bullet}$) from freshly fractured silica in the air.[17,18] These surface free radicals decayed with time after fracturing; that is, the $T_{1/2}$ for decay of the radicals on the fractured planes is ≈30 hours in air. These observations indicate that the reactivity of freshly fractured silica may be different from that of aged silica. Measurements of silica-induced toxicity and lipid peroxidation indeed show that freshly fractured silica is more toxic and more potent in inducing lipid peroxidation than aged silica.[18] These studies suggest that free radicals generated on the cleavage planes of silica may be involved in the mechanism of silica-induced cellular injury.

Using ESR spin trapping, our laboratories have shown that freshly fractured silica suspended in aqueous medium is able to generate $^{\bullet}OH$ radical.[18-20] The $^{\bullet}OH$ radical yield increases with prolonged grinding as the number of fractured planes increases. The $T_{1/2}$ for the ability of fractured silica to generate $^{\bullet}OH$ radical is ≈24 hours when the same silica particles are stored in air. Catalase, a scavenger of H_2O_2, inhibited the $^{\bullet}OH$ generation, while addition of H_2O_2 enhanced the yield, suggesting that H_2O_2 is a key intermediate in the $^{\bullet}OH$ generation. The generation of H_2O_2 by freshly fractured silica in aqueous media, depending on the pH and the temperature of hydrolysis, is high enough to be measured by a standard analytical chemistry method, the MnO_4^- reaction. ESR spin trapping studies also show that freshly fractured silica particles in aqueous media are able to generate superoxide radicals ($O_2^{\bullet -}$). In an aqueous suspension of freshly fractured silica, molecular oxygen is rapidly consumed, which appears to be the source of ROS generation by these silica reactions.[16]

Free radical generation from silica-stimulated cells. The generation of ROS represents one of the main mechanisms by which phagocytes kill invading organisms. ROS production increases in response to stimulation and/or phagocytosis of microorganisms, particulates, and chemicals, resulting in an increase in oxygen consumption called the "respiratory burst." Crystalline silica is a potent stimulant of the respiratory burst in alveolar macrophages.[20,21] The respiratory burst is associated with an elevated production of ROS as demonstrated by the following observations: (1) silica stimulates $O_2^{\bullet -}$ production; (2) H_2O_2 release from alveolar macrophages is increased following exposure to silica; and (3) enhanced chemiluminescence, an indication of ROS generation, is observed after in vitro exposure of macrophages to silica. In each of these cases, the effect of silica on respiratory burst is rapid, reaching a maximum within a few minutes of in vitro exposure. Enhancement of particle-induced generation of ROS has also been demonstrated using alveolar phagocytes harvested from rats exposed to silica.[22] Silica also stimulates the release of chemotactic agents, such as platelet-activating factor, macrophage inflammatory protein 2 (MIP-2), and cytokine-induced neutrophil chemoattractant (CINC) from alveolar macrophages.[23-25] These factors would result in the infiltration of neutrophils and/or monocytes into the air space, where the macrophage-derived platelet-activating factor and other mediators would stimulate oxygen metabolism, leading to further ROS generation.

NF-κB

This transcription factor is found in many different cell types and is involved in the transmission of signals from the cytoplasm to the nucleus. NF-κB regulates a variety of genes involved in inflammatory or acute phase responses, such as expression of various cytokines and surface receptors.[26-32] It may be noted that silica is a fibrogenic agent because of its ability to elicit resident macrophages to release inflammatory mediators and cytokines, which can promote fi-

broblast proliferation and collagen deposition. It has been suggested that NF-κB activation plays a crucial role in cytoplasmic/nuclear signaling, which occurs when cells are exposed to injury-producing conditions.[33] NF-κB serves as a mediator in inducing a series of cellular genes in response to environmental perturbations. Among the cellular genes regulated by NF-κB are several proinflammatory or fibrogenic cytokines, including IL-1, IL-2, IL-6, and TNF-α.[31] NF-κB activates these genes by acting as a transcriptional factor and binding to a NF-κB consensus sequence in their promoters. It has been suggested that ROS are mediators of NF-κB activation in response to a variety of initiators such as Cr(VI) or phorbol esters.[34-37] Because NF-κB is an oxidative stress response transcription factor, it is possible that signals for a variety of silica-induced responses are due to a common signaling component, which is regulated by ROS. In cells which have inducible NF-κB activity, the active form of this factor is composed of two different subunits, p50 and p65.[38] In resting cells, NF-κB is retained in the cytoplasm in an inactive form by binding to inhibitory proteins known as inhibitor-α and -β (IκBα and IκBβ) of NF-κB, p105 (precursor of p50) and p100 (precursor of p52). Upon activation with extracellular stimuli, these inhibitory proteins are proteolytically degraded or processed by proteasomes and certain proteases, which allows NF-κB to dissociate from the inhibitory proteins and then translocate into the nucleus in an activated form. This translocation initiates or regulates early response gene transcription by the binding of NF-κB to a decameric motif GGGRNNYYCC (κB elements) found in the promoter regions of cellular or viral genes.

Activation of NF-κB by Silica

Our laboratories have shown that silica can activate NF-κB in the mouse macrophage cell line, RAW 264.7 cells.[39] While only a p50/p65 or p52/p65 heterodimer could be detected in lipopolysaccharide (LPS)-stimulated cells, both heterodimers and a p50/p50 homodimer were detected in silica-stimulated cells. The silica-induced NF-κB activation was both dose- and time-dependent, with peak activation appearing at a silica dose of 100 μg/mL and an incubation time of 12 hours.

Role of ROS in Silica-Induced NF-κB Activation

Results obtained from our laboratories have demonstrated that ROS are involved in silica-induced NF-κB activation. Among the ROS, $^{\bullet}OH$ radicals may play a key role. The following experimental observations support this conclusion:

1. The ability of freshly fractured silica to generate $^{\bullet}OH$ radical is enhanced in the presence of H_2O_2 as demonstrated by spin trapping measurements.[14,18,20]
2. Catalase, a scavenger of H_2O_2, blocked the NF-κB activation.
3. SOD, which dismutates $O_2^{\bullet-}$ to generate H_2O_2, exhibited an opposite effect. It may be noted that earlier studies have shown that molecular oxygen was consumed to generate $O_2^{\bullet-}$ by silica suspensions.[12,40] Silica-induced DNA damage was inhibited in an argon atmosphere, indicating the role of molecular oxygen in ROS generation and silica-induced damage.[12,40] It appears that O_2 is reduced to $O_2^{\bullet-}$, which generates H_2O_2. H_2O_2 produces $^{\bullet}OH$ radical via a Fenton or Fenton-like reaction. [See Eq. (1) for ROS generation.]

$$O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH \qquad (1)$$

4. Metal ions, such as Fe(II) but not Fe(III), enhanced the NF-κB activation. It is known that Fe(II) generates $^{\bullet}OH$ from H_2O_2 via the Fenton reaction [Eq. (2)].

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH \qquad (2)$$

   Fe(III), on the other hand, is unable to generate $^{\bullet}OH$ radical without being first reduced to Fe(II).

5. The metal chelator, deferoxamine, also reduced the NF-κB activation. Deferoxamine chelates metal ions, such as Fe(II) or Fe(III), to make them less reactive toward H_2O_2 and thus attenuate the generation of $^{\bullet}OH$ radicals.
6. An $^{\bullet}OH$ radical scavenger, formate, inhibited silica-induced NF-κB activation.

Role of SiOH Groups in Silica-Induced NF-κB Activation

SiOH groups on the silica surface have been considered to be involved in silica-induced cellular damage.[41-44] Chemical modifications of the silica surface can be used to reduce toxicity in vitro and fibrosis in vivo. It is known that when silica particles are exposed to water, surface silicon-oxygen bonds (Si-O) are hydrated, resulting in the formation of SiOH groups. Poly(2-vinylpyridine-*N*-oxide) (PVPNO) is able to bind to SiOH groups. PVPNO has been shown to inhibit the silica-induced toxicity, to decrease and delay the development of silicosis in experimental animals and in humans, and to block the interaction of silica surface with phosphate groups of DNA in vitro.[44-47] It has been reported that PVPNO can inhibit silica-induced production of oxygen radicals in cells.[48,49] The ESR spin trapping measurements in an earlier study have demonstrated the inhibitory effect of PVPNO on $^{\bullet}OH$ generation by silica plus H_2O_2,[39] implying the involvement of SiOH groups in the generation of $^{\bullet}OH$ radical from H_2O_2 by silica. Our laboratories have shown that PVPNO decreased silica-induced NF-κB activation.[39] The inhibition may be due to the combination of two factors: (1) PVPNO can inhibit silica-induced $^{\bullet}OH$ radical generation, and (2) through binding with SiOH group on the silica surface, PVPNO may reduce the hydrogen bonding interaction between the silica surface and oxygen atomic sites on the cell membrane. While further study is required, it may be postulated that the inhibitory effect of PVPNO on silica-induced NF-κB activation may contribute to the inhibition of this chemical on the development of silicosis.

Our studies have also shown that deferoxamine reduced silica-mediated $^{\bullet}OH$ radical generation and attenuated silica-induced NF-κB activation. Deferoxamine is widely used for the prevention and treatment of iron overload.[50,51] It inhibits $^{\bullet}OH$ radical generation from H_2O_2 by transition metals, including Fe, Cr, and V.[52,53] A large dose of deferoxamine (50 mg/kg/d) can be safely injected into humans.[51] Thus, further investigation is warranted on the use of deferoxamine or other metal chelators as a possible preventive strategy against silica-induced fibrosis and carcinogenesis.

Effect of Aspirin on Silica-Induced NF-κB Activation

Aspirin is a widely prescribed drug used to treat inflammation. Long-term use of aspirin in humans has also been reported to protect against the development of colon cancer and other digestive system cancers, including cancers of the esophagus and stomach.[54,55] Animal studies have shown that aspirin can inhibit chemically induced tumors of the colon, tongue, esophagus, pancreas, bladder, breast, and skin, as well as various sarcomas.[56,57] Recent studies from our laboratories have shown that aspirin functions as an antioxidant.[58] Because oxidative stress is involved in NF-κB activation, it is likely that aspirin may inhibit silica-induced NF-κB activation. Our studies have shown that aspirin is indeed able to inhibit silica-induced NF-κB activation,[39,58] suggesting that aspirin may be used to prevent or attenuate silica-induced disease.

Effect of Dexamethasone on Silica-Induced NF-κB Activation

The intratracheal instillation of silica into male F-344 rats led to an increase in PMNs in BAL fluid.[59] This increase was prevented by treatment with dexamethasone. In silica-treated rats, activation of NF-κB was detected in BAL cells beginning 1 hour after instillation and was apparent as long as 18 hours after instillation. Dexamethasone treatment reduced this silica-induced NF-κB expression in BAL cells. This reduction was approximately 70%. Dexamethasone, an anti-inflammatory steroid, acts by binding to cells with a steroid receptor in the cytoplasm that translocates to the nucleus and blocks the promoter site of κB promoters. This prevents the NF-κB dimer from being able to bind to DNA and upregulate transcription of the various inflammatory genes. Alternatively, it may prevent degradation of IκBα, a process that would prevent activation of NF-κB.

Effect of Tetrandrine on Silica-Induced NF-κB Activation

Tetrandrine has been reported to retard and reverse the fibrotic lesions of silicosis in humans and in rats,

but its mechanism of action is unclear.[60,61] It has been reported that tetrandrine is an effective inhibitor of silica-induced H_2O_2 production by macrophages.[62] In addition, recent studies have shown that tetrandrine is capable of scavenging $^{\cdot}OH$ radicals and inhibiting silica-induced lipid peroxidation.[13] Tetrandrine has been shown to be an effective inhibitor of IL-1 secretion from alveolar macrophages activated by silica or LPS.[62] We have demonstrated that tetrandrine inhibited silica, PMA, and LPS-induced NF-κB activation.[39,63] It appears that the mechanism of NF-κB activation induced by LPS, although it can be inhibited by tetrandrine, is different from that of silica. In the case of silica, both ascorbate and formate exhibited inhibitory effects. For LPS, the effect of these antioxidants is weak. Although it is likely that one of the steps involves antioxidant activity of tetrandrine toward $^{\cdot}OH$ radical, other mechanisms may also exist. For example, tetrandrine decreases the stimulant-induced increase in intracellular calcium concentration resulting from its channel-blocking effect and inhibits the production of nitric oxide (NO) in activated macrophages.[64] The latter is especially important, since the production of NO has a direct effect on silica-induced NF-κB activation.[65]

Role of NF-κB in Silica-Induced Cyclooxygenase II

Products of arachidonic acid metabolism are critical participants in the development of the inflammatory response after infection or tissue injury. Prostaglandin E_2 (PGE_2) is one of the most studied mediators of this process.[66] The cyclooxygenase (COX) enzyme is considered to be the rate-limiting step for PGE_2 formation. Mammalian cells contain two related isoforms of COX, referred to as COX I and COX II, that are encoded by separate genes.[67] The latter is expressed after exposure to mitogenic or pathological stimuli, including cytokines, phorbol esters, and bacterial endotoxin. By virtue of its early induction after stimulation by inflammatory agents, COX II has been included in the early response gene family.[68] Although induction of COX II gene expression has been demonstrated, the mechanism for this induction remains to be investigated. The NF-κB consensus motifs or similar motifs were found in the COX II genes for nearly all of the species tested.[69] Because NF-κB is an important transcription factor involved in the expression of early response genes related to several inflammatory mediators after extracellular stimuli, it is possible that NF-κB may be crucial for silica-induced COX II gene transcription. Our laboratories have shown that suppression of NF-κB activation in these cells leads to an attenuation of COX II mRNA accumulation induced by silica.[70] At least two κB sites in the 5′-flanking region of the rat COX II gene are involved for silica-induced transcriptional control of the COX II gene. The first motif, −404 GGGGATTCCC -395, is absolutely conserved in sequence and is located in a similar position among the COX II genes found in humans, rats, and mice. The second motif, −91 GGGGAAAGCC −82, was conserved only in the mouse and rat COX II genes in sequence and in location. Aspirin, a COX inhibitor, suppressed silica-induced NF-κB activation. PGE_2, one of the downstream reaction products catalyzed by the COX enzyme, was also shown to inhibit silica-induced NF-κB activation by retarding the degradation of NF-κB inhibitor.[70] Therefore, PGE_2 may act through a negative feedback on NF-κB to modulate the production of inflammatory mediators and growth factors under the NF-κB transcriptional control. Indeed, it has been sugested that a shift from the cyclooxygenase to the lipoxygenase pathway of arachidonic acid metabolism is important in the progression of silicosis.[71]

The fact that PGE_2 and COX II inhibitors affect NF-κB activation is of great interest. Although aspirin can block signal-induced IκBα degradation and subsequent NF-κB activation, the effective target of aspirin seems to be nonspecific. For example, we have found that aspirin may inhibit NF-κB activation via its function as an antioxidant.[39] With regard to PGE_2, it has been shown that PGE_2 inhibits immune cell proliferation and downregulates several cytokines, including IL-1, IL-2, and interferon-γ.[72] The inhibitory effects of PGE_2 on immune function may be partially linked to its ability to suppress the degradation of IκBα and consequently block the cytoplasmic/nuclear translocation of NF-κB that is essential for transcriptional regulation of gene expression of certain cytokines. In the case of silica-induced inflammatory gene response, the negative feedback role of PGE_2 on NF-κB activation may benefit host self-protection by tempering the positive feedback loop between the production of cytokines and silica-induced NF-κB activation.

Role of NF-κB in Silica-Induced TNF-α Production

Several studies have indicated that macrophage cytokines, most notably TNF-α, play an important role in the development of silicosis. Silica is able to stimulate TNF-α release and upregulate its mRNA expression in macrophages.[73] Antisense inhibition of TNF-α mRNA expression causes a corresponding decrease in silica-induced TNF-α production.[74] The role of TNF-α as a key mediator of silicosis has further been demonstrated by the ability of anti-TNF-α antibodies and exogenous recombinant TNF-α to attenuate and augment, respectively, silicotic fibrosis.[75]

The expression of TNF-α is regulated at several levels: transcriptional, post-transcriptional, translational, and post-translational. It is generally believed that the regulation of the TNF-α occurs primarily at the transcription level. Several transcription factors including NF-κB, AP-1, AP-2, NFAT, Erg-1, C/EBPβ, Ets, and CREB have been reported to activate TNF-α gene promoter. The activation of the TNF-α promoter by different transcription factors is dependent on the nature of the stimulation and on the cell type investigated. For example, NF-κB, but not AP-1 or AP-2, is involved in the activation of TNF-α transcription of LPS-stimulated monocytes, whereas NFAT, but not NF-κB, plays a role in PMA-stimulated T cells.[76]

We have recently investigated the role of NF-κB and oxygen free radicals in silica-induced TNF-α production in primary alveolar macrophages and in RAW 264.7 cells.[66] We have demonstrated that the inhibition of NF-κB activation by SN50, a specific NF-κB blocker, abolishes silica-induced TNF-α production.[77] Pretreatment of the cells with catalase (an H_2O_2 scavenger) or deferoxamine (a metal chelator) to reduce $^{\bullet}OH$ generation effectively inhibits NF-κB activation and TNF-α production, whereas superoxide dismutase (an $O_2^{\bullet-}$ scavenger that generates H_2O_2) has an opposite effect. These results indicate that silica-mediated free radical generation and NF-κB activation may play important roles in silica-induced TNF-α gene expression.

Conclusions

Freshly fractured silica is able to generate free radicals by its direct interaction with aqueous medium and by its stimulation of cells. Among the free radicals generated by silica, $^{\bullet}OH$ radicals play a key role in silica-induced NF-κB activation. SiOH groups on the silica surface are also involved, providing a favorable environment for the interaction of silica with the cell membrane. Aspirin, dexamethasone, and tetrandrine are able to attenuate silica-induced NF-κB activation. NF-κB is involved in silica-induced generation of cyclooxygenase and TNF-α. Although it is not discussed in this article, recent studies have also shown that silica is able to cause (1) activation of AP-1, (2) induction of various MAP kinase family members, (3) upregulation of p53, (4) activation of various protein kinases, and (5) cell apoptosis. Further studies on the molecular mechanisms of silica-induced lung injuries and the role of ROS in these events may help develop therapeutic agents to prevent or attenuate silica-induced silicosis and carcinogenesis.

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