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Original Research Communication

Is the Effect of Interleukin-1 on Glutathione Oxidation in Cultured Human Fibroblasts Involved in Nuclear Factor-κB Activation?

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ABSTRACT

Our understanding of the interleukin-1 (IL-1) signaling molecular mechanisms has recently made considerable progress, with the discovery of the IL-1 receptor-associated kinase and the downstream enzymatic cascade that leads to the activation of nuclear factor-κB (NF-κB). IL-1 signaling and especially NF-κB activation are thought to be redox-sensitive, even though the precise nature and the molecular targets of the oxidants/antioxidants involved remain largely unknown. Here, we investigated the possible role of cellular oxidized/reduced glutathione (GSSG/GSH) balance in IL-1 signaling. We describe a quantitative method based on capillary electrophoresis designed to assay both intracellular GSH and GSSG in adhering fibroblasts. This method allows the GSSG/GSH balance to be followed during IL-1 stimulation. Our data show that IL-1 induces rapid and transient oxidation of intracellular glutathione in human fibroblasts. Using various antioxidants, including pyrrolidine dithiocarbamate and curcumin, we were unable to show a direct relationship between this IL-1–induced glutathione oxidation and NF-κB activation. Of the five antioxidants tested, only curcumin was able to inhibit IκBα degradation upstream and, hence, NF-κB DNA-binding activity and NF-κB-dependent expression of IL-6 downstream. Antioxid. Redox Signal. 3, 329–340.

INTRODUCTION

INTERLEUKIN-1 (IL-1) is a powerful proinflammatory cytokine, mediating many of its biological effects through the activation of the transcription factor nuclear factor-κB (NF-κB). In resting cells, this dimeric transcription factor is inactive, sequestered in the cytoplasm by an inhibitory protein called IκB (3). Cell stimulation by IL-1 or by other proinflammatory mediators like tumor necrosis factor-α (TNFα) triggers the activation of the IκB kinases (IKK) responsible for the phosphorylation of IκB. This phosphorylation is followed by a ubiquitin-dependent degradation of IκB by the 26S proteasome (for review, see 29). NF-κB is consequently released from its cytoplasmic anchor (IκB), enters into the nucleus, and induces the transcription of a large number of genes encoding immune response mediators. The transcription factor NF-κB can thus be considered as a major actor in the cell response to infections (for review, see 2).

Concomitantly, it is generally accepted that prooxidant conditions favor NF-κB activation (for a review, see 15). This is based on three observations. First, by themselves oxidative stresses, like reoxygenation (37), hyperoxia

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(43), or incubations in the presence of hydrogen peroxide (H_2O_2) (39), are able to induce NF-κB activation, at least in some cell types (1). Nevertheless, the molecular mechanisms leading to NF-κB activation appear to be different in the case of physiological stimuli like IL-1 and H_2O_2 (6). Second, NF-κB activation can be inhibited by a wide variety of antioxidants, especially by metal chelators or -SH protecting molecules (15, 39). Modulation of intracellular antioxidant enzymes by chemical inhibitors decreases their activities, whereas transfection leads to their overexpression, which in turn affects NF-κB activation (23, 35). Third, some authors have reported the presence of oxygen-derived species in cells stimulated by various NF-κB inducers. Even though this has been clearly shown for TNFα (40), the role of oxygen-derived species in IL-1–dependent signal transduction is poorly documented. Primary human fibroblasts (28) and human mesangial cells (33) release superoxide anions in the medium after exposure to IL-1. To our knowledge, the only measurement of intracellular reactive oxygen species production after IL-1 stimulation was made by Tiku and co-workers, who detected H_2O_2 in rabbit articular chondrocytes (47).

Several data indicate that oxidized glutathione (GSSG) may participate in the activation mechanism of NF-κB. Bischloroethylnitrosourea, an inhibitor of glutathione reductase that elevates the intracellular GSSG/reduced glutathione (GSH) ratio, enhances the NF-κB DNA-binding activity induced by IL-1 (35), TNFα (42), or phorbol myristate acetate (16). In addition, several GSH-increasing compounds tend to inhibit NF-κB activation (for a review, see 15). These data suggest that GSSG facilitates NF-κB activation, although high concentrations of GSSG can inhibit the DNA-binding activity of NF-κB (16) due to the oxidation of a particular cysteine residue located in the DNA-binding domain of NF-κB (27). Consequently, GSSG appears to influence the DNA-binding activity of NF-κB in a bell-shaped manner, as certain amounts of GSSG seem to be required for NF-κB activation in the cytoplasm, whereas an excess of GSSG inhibits the DNA-binding activity of NF-κB in the nucleus (12).

TNFα and phorbol ester, two major activators of the transcription factor NF-κB, have been shown to induce a decrease of cellular GSH (44); however, as yet nothing is known regarding IL-1 and a possible change in the redox status of glutathione. Here, we investigated the relationship between the level of GSH and GSSG and the IL-1 activation. We adapted a method based on capillary electrophoresis (32) to show that IL-1 triggers a weak and transient oxidation of glutathione. By using various antioxidant molecules, we further investigated the role of this GSSG/GSH ratio modification in IL-1–induced NF-κB activation at three different levels: IκBα degradation, NF-κB DNA-binding activity, and the gene expression of interleukin-6 (IL-6) which is controlled by NF-κB. Our data show that antioxidants affect regulation, but the mechanisms involved are clearly much more intricate than previously shown, and also that the effects differ from one antioxidant to the other.

MATERIALS AND METHODS

Cell culture

The cell line WI-38 VA13, an SV40 virus-transformed human fibroblast cell line, was purchased from the American Type Culture Collection and plated in 25- or 75-cm^2 flasks at 60,000 cells/cm^2. Cells were serially cultivated in minimum essential medium (GIBCO, U.K.) supplemented with 10^{-7} *M* Na_2SeO_3 and 10 m*M* HEPES in the presence of 10% fetal bovine serum. For each experiment, cells were rinsed for at least 1 h in the same medium without serum, but in the presence of 0.2% lactalbumin hydrolysate as serum substitute. They were then incubated in the same medium with H_2O_2 (Merck, Darmstadt, Germany), IL-1β (R&D Systems Inc., Minneapolis, MN, U.S.A.), and/or several antioxidants: pyrrolidine dithiocarbamate (PDTC), curcumin, and Trolox C purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.), taxifolin, and L-ergothioneine from Calbiochem (U.K.), and *N*-ethylmaleimide from Janssen Chimica (Belgium). Before being diluted in culture medium, Trolox

C was resuspended in ethanol, and curcumin and taxifolin were resuspended in dimethyl sulfoxide. The other molecules were directly resuspended in culture medium. Ethanol and dimethyl sulfoxide concentrations never exceeded 0.5%. None of these molecules were toxic for cells at the concentrations used (data not shown).

Glutathione assay by capillary electrophoresis

Piccoli's method for red blood cells (32) was adapted for adherent fibroblasts. Cells grown to confluence in 75-cm^2 flasks were stimulated for various amounts of time and then rinsed twice with cold phosphate-buffered saline. They were harvested with a rubber policeman in 400 μl of 25 m*M* *N*-ethylmaleimide dissolved in a 10 m*M* phosphate buffer (pH 7.4) and homogenized with a Dounce. The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were collected and centrifuged for 30 min at 2,000 rpm at 4°C on an Ultrafree-4 filter (Biomax 10 K, Millipore, Bedford, MA, U.S.A.) to remove proteins larger than 10 kDa. The filtrates were then analyzed by electrophoresis on a 50-cm P150 capillary of 50 μm diameter (Supelco, Bellefonte, PA, U.S.A.). The sample was injected under 40-mbar pressure for 0.4 min and then migrated in a 35 m*M* phosphate buffer (pH 2.1) under an 18 kV voltage (field: 360 V/cm; intensity: 30 μA). Detection was performed at 200 nm on a Prince system (Lauerlabs, The Netherlands). For standard curves, purified GSSG and GSH from Sigma (St Louis, MO, U.S.A.) were dissolved in a 10 m*M* phosphate buffer (pH 7.4) containing 25 m*M* *N*-ethylmaleimide.

Electrophoretic mobility shift assay

Confluent cells in 25-cm^2 flasks were preincubated for 1 h with various antioxidant molecules before the addition of IL-1 at a final concentration of 2.5 ng/ml. After a 30-min stimulation with IL-1, cells were rinsed twice with cold phosphate-buffered saline, scraped, and centrifuged for 10 min at 1,000 rpm. The pellet was then resuspended in 100 μl of lysis buffer (20 m*M* HEPES, 0.35 *M* NaCl, 20% glycerol, 1% NP-40, 1 m*M* $MgCl_2 \cdot 6H_2O$, 0.5 m*M* EDTA, 0.1 m*M* EGTA) containing a cocktail of phosphatase and protease inhibitors (Boehringer, Mannheim, Germany). After 10 min on ice, the lysate was centrifuged for 20 min at 13,000 rpm. The supernatants were kept frozen at −70° C. The probe binding reaction was carried out in 20 μl of binding mixture containing 2 m*M* HEPES (pH 7.5), 5% glycerol, 75 m*M* KCl, 2.5 m*M* dithiothreitol, 2 μg of poly(dIC), 20 μg of bovine serum albumin, cell extract (25 μg of protein), and a ^{32}P-labeled oligonucleotide (±1 ng or 20,000 cpm). The cold double-stranded probe containing the NF-κB consensus sequence (5′-AGTTGAGGGGACT-TTCCCAGGC) was purchased from Promega (Madison, WI, U.S.A.), labeled with γ-^{32}P using the T4 polynucleotide kinase, and purified on a Sephacryl S-200 column. After 30 min of incubation, the binding mixture was analyzed on a nondenaturating 4% acrylamide gel in 0.5 × TBE (0.9 *M* Tris. 0.9 *M* boric acid, 0.02 *M* EDTA). After autoradiography, the film was scanned by an image analysis system (Visage 101, Millipore) that allows quantification of the optical density corresponding to the shifted band. NF-κB in the WI-38 VA13 cells stimulated with IL-1 has been previously identified as p50–p65 by supershift experiments (35).

IκB degradation

Cytoplasmic extracts were prepared as described (36) by cell lysis in hypotonic buffer [50 m*M* NaCl, 10 m*M* HEPES (pH 8.0), 500 m*M* sucrose, 1 m*M* EDTA, 0.2% Triton] containing a cocktail of protease and phosphatase inhibitors (Boehringer). Lysates were centrifuged at 5,000 *g* for 5 min to sediment nuclei. Cytoplasmic extracts were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride Immobilon membranes (Bio-Rad, Hercules, CA, U.S.A.) by semidry electroblotting. Western blotting was then performed as described (36) using anti-human IκBα rabbit and anti-α-tubulin mouse antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Immobilized antigen–antibody complexes were detected with secondary anti-IgG–horseradish peroxidase conjugates and an enhanced chemilumi-

nescence detection system (NEN, Boston, MA, U.S.A.).

IL-6 assay

Cells were cultivated in 96–well plates at a density of 5,000 cells/well in 100 μl of minimum essential medium plus 0.2% lactalbumin hydrolysate. They were preincubated for 1 h with various antioxidants before the addition of 2.5 ng/ml IL-1β (final concentration). IL-6 was assayed by ELISA (Eurogenetics, Tessenderlo, Belgium) in the culture medium collected after 6 h of stimulation with IL-1.

Statistical treatment

Results are presented as means ± SD Statistical analysis were performed using one-way analysis of variance with Scheffé's contrasts for multiple comparison.

RESULTS

Simultaneous measurement of purified GSSG and GSH

Before applying the capillary electrophoresis-based assay of glutathione on cell extracts, the assay was initially checked on purified glutathione. When GSSG and GSH were mixed and assayed by capillary electrophoresis as described by Piccoli *et al.* (32), three peaks appeared in the electrophoretic pattern (Fig. 1). To identify the different peaks, GSSG alone was injected and its migration time corresponded to the first peak. Similarly, injection of purified GSH led to the identification of the second peak as GSH bound to *N*-ethylmaleimide, whereas the third peak corresponded to free GSH. This third peak is present when GSH is in excess to *N*-ethylmaleimide. If the sample is left overnight at room temperature before being analyzed, this third peak disappears and the first one increases, indicating that unprotected GSH is rapidly oxidized.

In addition, when GSH and GSSG were injected at identical concentrations and under the same experimental conditions, the integration of the peak corresponding to GSSG led to values that were systematically 2.4 times lower than the ones obtained for the GSH peak protected by *N*-ethylmaleimide. This apparent discrepancy can easily be explained as the binding of *N*-ethylmaleimide to GSH increases the absorbance properties of GSH at 200 nm. Consequently, a correction factor was further introduced in the calculations: the value corresponding to GSH was based on the integration of the second peak divided by 2.4, plus the integration of the third peak, whenever present, because free GSH and GSSG display the same absorbance coefficient. Using the correction factor, concentration curves of GSH and GSSG standard solutions were established. The different peaks were integrated and the values plotted against the glutathione concentration (Fig. 2). For both GSH and GSSG, a linear relationship was obtained between the values obtained after peak integration and the glutathione concentration, ranging from 0 to 200 μM.

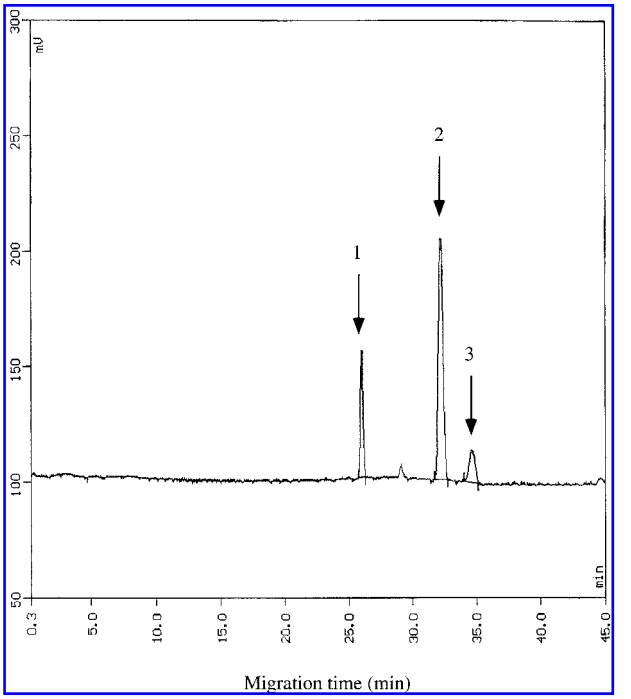

FIG. 1. Electropherogram of glutathione. Sixty micrograms of purified GSSG and 60 μg of purified GSH were dissolved in 1 ml of 10 m*M* phosphate buffer (pH 7.5) containing 25 m*M* *N*-ethylmaleimide. This solution was analyzed by capillary electrophoresis as described in Materials and Methods. Peaks indicated by arrows correspond to GSSG (1), GSH bound to *N*-ethylmaleimide (2), and free GSH (3).

Measurement of intracellular GSSG and GSH

The glutathione assay method, previously developed for circulating red blood cells (32),

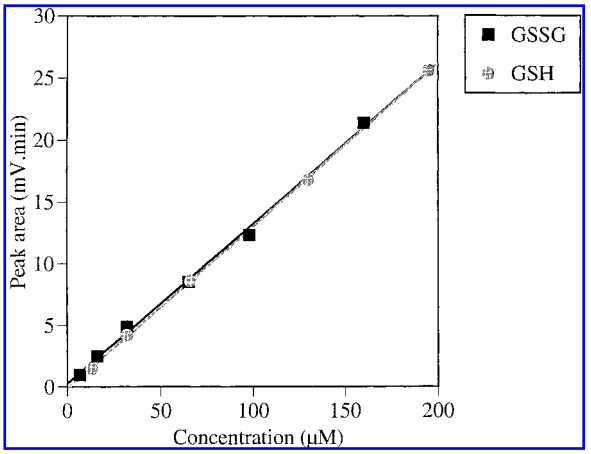

FIG. 2. Calibration curve of GSSG (black) and GSH (gray) standard solutions. Different concentrations of purified GSSG and GSH were prepared and analyzed by capillary electrophoresis as described in Fig. 1. The integrated areas corresponding to GSSG and GSH were calculated and plotted against the concentration. The GSH peak area was divided by the correction factor 2.4 as explained in the text.

was then adapted for adhering WI-38 VA13 fibroblasts cultured *in vitro*. Immediately after the stimulatory treatment, cells were scraped in the presence of *N*-ethylmaleimide to avoid any GSH oxidation during the extraction procedure. The method was first tested on fibroblasts stimulated with H_2O_2, which is known to oxidize intracellular glutathione (34). An electropherogram from fibroblasts stimulated for 10 min with 0.5 m*M* H_2O_2 is presented in Fig. 3. The two peaks obtained were identified as GSSG and GSH, as confirmed by injection of purified GSSG and GSH (data not shown).

The intracellular GSSG content present in fibroblasts increased in a dose-dependent manner in correlation to the H_2O_2 concentration used: after a 10-min incubation, the GSSG content, expressed as the percentage of total glutathione, reached 7, 23, and 41% in the presence of 0.2, 0.5, and 1 m*M* H_2O_2 respectively (Table 1).

The GSSG content was then followed as a function of time in cells incubated with 0.5 m*M* H_2O_2 (Fig. 4). After 5 min, the GSSG content reached 70% of the total intracellular glutathione, and then decreased exponentially to recover basal levels after 1 h (~1% of total glutathione content). In unstimulated cells, GSSG was barely detectable, whereas the GSH peak was prominent. From the calibration curve (Fig. 2), the glutathione content in these human fibroblasts was estimated to be 3.2 $\mu g/10^6$ cells, which is in agreement with previous data on adipose cells (19).

Once validated, the glutathione assay was then used to follow cells activated with IL-1. Figure 5 shows that IL-1 quickly induces a slight increase in the GSSG content, reaching a peak of ~3.7% after 5 min. This increase is very transient, as cells recover their basal level 10 min later. To our knowledge, this is the first time that glutathione oxidation following IL-1 stimulation in human cells has been demonstrated.

Effect of various antioxidants on IL-1-induced NF-κB activation

To investigate if the GSH oxidation induced by IL-1 stimulation is linked to NF-κB activation, we used PDTC, an antioxidant widely used to inhibit NF-κB activation (38). First, we tested if this molecule could inhibit IL-1–induced NF-κB activation in WI-38 VA13 fibroblasts. Three levels of NF-κB activation were studied: (i) the degradation of IκBα, evaluated by western blotting; (ii) the binding of NF-κB to DNA measured by gel retardation; and (iii) the expression of the IL-6 gene directly controlled by NF-κB. PDTC dose-dependently in-

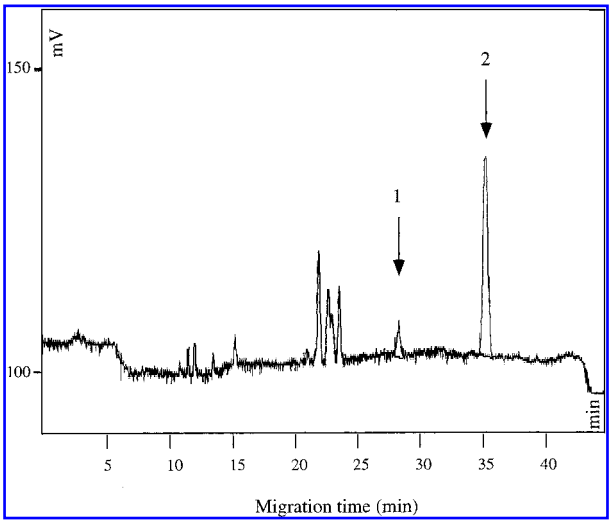

FIG. 3. Electropherograms of fibroblasts stimulated with H_2O_2 (field: 360 V/cm; intensity: 30 μA). Fibroblasts were stimulated for 10 min with 0.5 m*M* H_2O_2 and then treated for capillary electrophoresis analysis as described in Materials and Methods. Peaks indicated by arrows correspond to GSSG (1) and GSH bound to *N*-ethylmaleimide (2).

TABLE 1. EFFECTS OF H_2O_2 ON GLUTATHIONE OXIDATION

Treatment	*GSSG (% of total glutathione)*
Untreated	1.2
H_2O_2 (0.2 m*M*)	7
H_2O_2 (0.5 m*M*)	23
H_2O_2 (1.0 m*M*)	43

Cells were incubated for 10 min in the presence of H_2O_2 at the different concentrations indicated. GSH and GSSG were estimated as described above and GSSG expressed as the percentage of total glutathione (GSSG + GSH).

hibited IL-1–induced NF-κB binding to DNA (Fig. 6B) and IL-1–induced IL-6 synthesis (Fig. 6C) as previously described (35). PDTC alone did not affect NF-κB DNA-binding capacity (data not shown). Unexpectedly, IL-1–induced IκBα degradation was not affected by PDTC (Fig. 6A). This apparent discrepancy may be because the fact that PDTC can, in some conditions, exert a prooxidant effect in the nucleus, which explains the inhibition of the DNA-binding capacity of NF-κB (9). If, as previously expected, PDTC exerts its inhibitory effect at the nuclear level rather than at the cytoplasmic level, it then becomes pointless to try to relate the PDTC's inhibitory effect with IL-1–induced GSH oxidation. Curcumin (diferulomethane) presents antiinflammatory properties, partly due to the inhibition of NF-κB activation (24), however so far no oxidant properties are known. Figure 7 shows that curcumin also dose-dependently inhibits IL-1–induced NF-κB activation at all three levels studied: IκBα degradation (Fig. 7A); NF-κB DNA-binding capacity (Fig. 7B), and IL-6 release (Fig. 7C). As curcumin inhibits NF-κB activation in WI-38 VA13 fibroblasts, we measured the oxidation level of glutathione in the presence of increasing concentrations of this molecule (Fig. 8). Unexpectedly, curcumin alone induced a dose-dependent increase in the cellular GSSG content: in the presence of 100 μ*M* curcumin, GSSG represents 9.2% of the total glutathione content, whereas the maximum value obtained when cells are stimulated with IL-1 is 3.7% (see Fig. 5). This result is not due to direct inhibition of glutathione reductase (data not shown). To rule out the possibility that curcumin oxidizes GSH during the extraction procedure, the effect of curcumin on purified glutathione was tested. Purified GSH or GSSG (0.5 m*M*) resuspended in serum-free culture medium was incubated for 1 h at 37°C in the presence of increasing concentrations of curcumin. When curcumin was incubated in the presence of pure GSH, no GSSG was detectable (data not shown), indicating that GSH is not oxidized during the extraction procedure. Conversely, when curcumin was incubated in the presence of pure GSSG, ~1% of GSSG was reduced into GSH. Similar results were obtained when supernatants of cells incubated for 1 h with curcumin were then incubated in the presence of purified GSSG or GSH (data not shown). These results suggest that although curcumin can be considered as an antioxidant *in vitro*, it can also lead to the oxidation of intracellular GSH.

As curcumin alone induces a nonnegligible oxidation of intracellular glutathione, it was not possible to study its effect on the IL-1–induced glutathione oxidation. Four other molecules were then tested on IL-1–induced NF-κB activation: (i) taxifolin (dihydroquercetin), an antioxidant flavonoid protecting mammalian cells from hydroperoxide- and superoxide anion-induced cytotoxicity (30); (ii) Trolox C, a water-soluble vitamin E analogue protecting cells against free radical-mediated membrane damage (11); (iii) ergothioneine, a natural thiol-containing molecule with antioxidant properties, which can also scavenge several free rad-

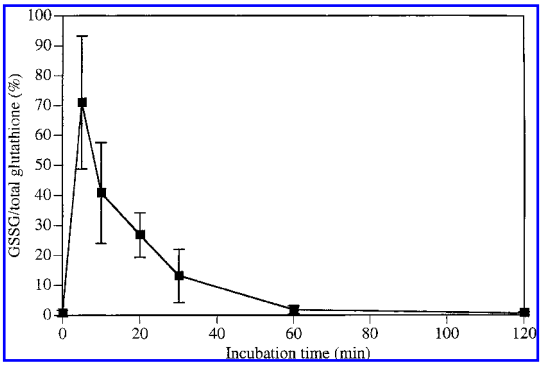

FIG. 4. Effect of H_2O_2 on the intracellular GSSG content, expressed as % of total glutathione. Fibroblasts were stimulated for 0, 5, 10, 20, 30, 60, or 120 min with 0.5 m*M* H_2O_2 and then treated for capillary electrophoresis analysis. In the resulting electropherograms, the peak areas corresponding to GSSG and GSH were integrated, and the GSSG content was deduced from these values as described in Table 1. Values represent the means ± SD of three independent experiments.

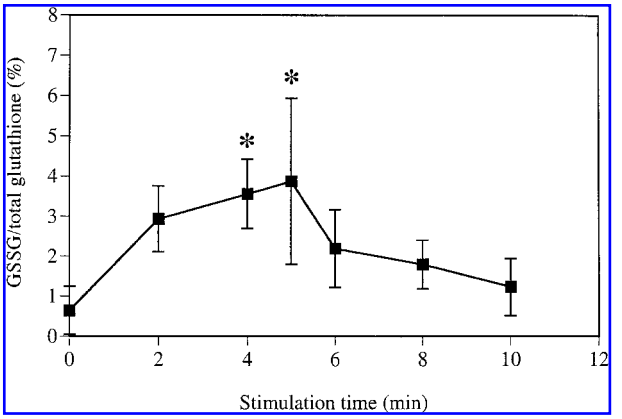

FIG. 5. Effect of IL-1 on the intracellular GSSG content, expressed as % of total glutathione. Fibroblasts were stimulated for 2, 4, 5, 6, 8, or 10 min with 5 ng/ml IL-1β (IL-1). Cells were then treated for capillary electrophoresis analysis as described in Materials and Methods. The resulting electropherograms were analyzed as previously described. Values represent the means ± SD of four experiments. *Significantly different from the control (time 0) with $p < 0.05$.

ical molecules, block lipid peroxidation, and chelate divalent metal ions (20); and (iv) *N*-ethylmaleimide, which is not considered as an antioxidant but irreversibly alkylates free sulfhydryls and inhibits the DNA-binding capacity of NF-κB (48). Table 2 summarizes the results obtained on NF-κB activation at the three steps studied. When an inhibitory effect was observed, it was dose-dependent. The percentage of inhibition is indicated in parentheses only for the highest concentration tested. Our data showed that the antioxidant molecules can be classified into four classes: (i) molecules that inhibit NF-κB activation at all three levels (curcumin); (ii) molecules that inhibit DNA-binding activity and IL-6 production (PDTC and *N*-ethylmaleimide); (iii) molecules that inhibit only IL-6 production (taxifolin); and (iv) molecules with no detectable effects at the concentrations tested (Trolox C, ergothioneine).

DISCUSSION

It is largely accepted that IL-1 induces intracellular oxidative events, probably involved in signal transduction, because antioxidants can inhibit the IL-1–induced expression of several molecules, or the phosphorylation of mitogen-activated protein kinase. Nevertheless, only one group has effectively measured intracellular reactive oxygen species following IL-1 stimulation, showing an elevation of the H_2O_2 level in rabbit articular chondrocytes (47). As several of the antioxidants are known to inhibit NF-κB activation, and have been reported to interfere with glutathione metabolism, one could speculate that IL-1 provokes a modification of the glutathione redox balance.

Measurement of GSSG is hampered by its limited abundance and by the rapid oxidation of GSH in biological materials (5). To detect slight changes of the GSSG/GSH balance in cells, we adapted a method described by Piccoli *et al.* (32), where GSH and GSSG can be

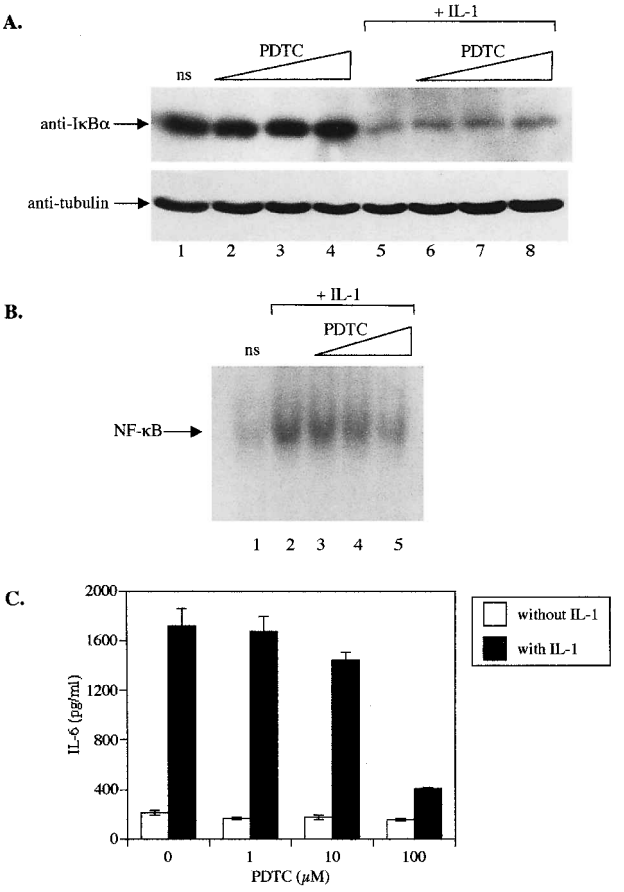

FIG. 6. Effect of PDTC on IL-1-induced IκB degradation (A), NF-κB DNA-binding capacity (B), and IL-6 synthesis (C). Fibroblasts were preincubated for 1 h with or without increasing concentrations of PDTC (1, 10, and 100 μM) before the addition of IL-1 for 20 min in the case of western blot (A), 30 min for gel retardation (B), or 6 h for ELISA (C). The data presented in C are the means ± SD of four values. ns, not stimulated.

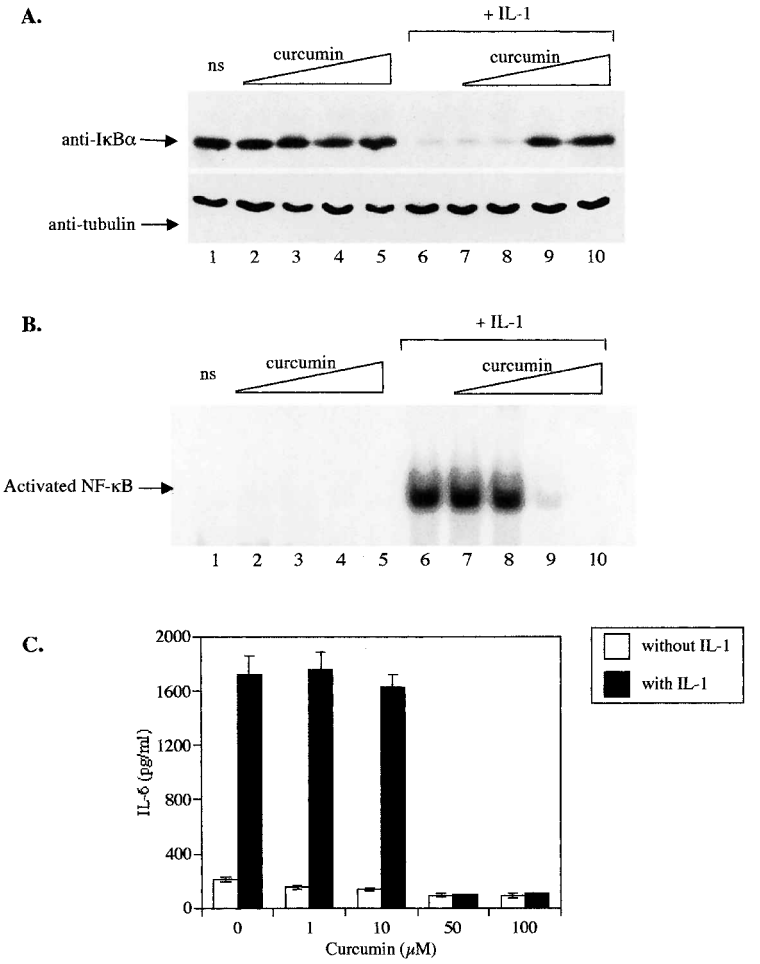

FIG. 7. Effect of curcumin on IL-1-induced IκB degradation (A), NF-κB DNA-binding capacity (B), and IL-6 synthesis (C). Fibroblasts were preincubated for 1 h with or without increasing concentrations of curcumin (1, 10, 50, and 100 μM) before the addition of IL-1 for 20 min in the case of western blot (A), 30 min for gel retardation (B), or 6 h for ELISA (C). ns, not stimulated. The data presented in C are the means ± SD of four values.

measured simultaneously by capillary electrophoresis. Using this technique, an increase in the GSSG content in IL-1–stimulated fibroblasts, reaching 3.7% of total glutathione after 5 min and returning to a basal ratio of 0.6% after 10 min (Fig. 5), was detected for the first time.

Although the oxidation of GSH induced by IL-1 is quite weak compared with H_2O_2 treatment, it does not minimize its possible role in IL-1 signaling. Indeed, the 3.7% GSSG content measured reflects the global cytoplasmic change in the GSSG/GSH balance, but one cannot exclude that higher ratios could be locally achieved, for example, near the plasma membrane where the IL-1 receptor is located. In addition, the rapid and transient character of IL-1–induced GSH oxidation is compatible with the kinetics of signal transduction usually triggered by IL-1, and leading to the phosphorylation of IκB.

The concept of reactive oxygen species as second messengers in signal transduction is not new (for a review, see 14). Further, GSSG is a particularly good candidate for the role of "redox sensor" in signal transduction (41), as it is ubiquitous, can diffuse quickly, and can be rapidly produced in response to an extracellular signal by oxidizing a part of the large intracellular pool of GSH. In addition, several enzymes are already known to be activated or inhibited by GSSG (12). Several mechanisms have been proposed to relate enzymatic activity with oxidation of the environment. First, en-

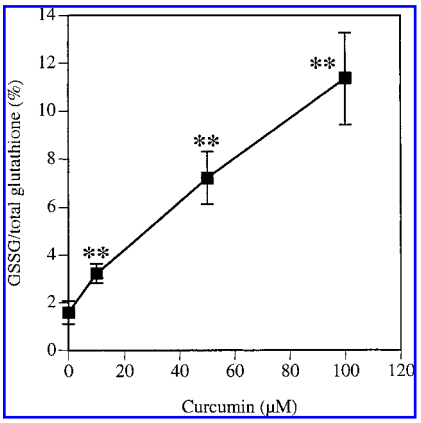

FIG. 8. Effect of curcumin on the intracellular GSSG content, expressed as % of total glutathione. WI-38 VA13 fibroblasts were incubated for 1 h in serum-free medium containing increasing concentrations of curcumin. Cell extracts were then prepared as described to assay GSSG and GSH by capillary electrophoresis analysis. Values are the means ± SD of three experiments. **Significantly different from the control with $p < 0.01$.

zymatic activity could be enhanced by the formation of intermolecular (4) or intramolecular disulfide bridges (21). Second, enzymes like protein tyrosine phosphatases have cysteines in their active site, and modifications of the redox environment can influence their catalytic activity (17). This is the case for a phosphatase activated by TNFα or IL-1 (18). At least another enzyme activated by IL-1 has already been shown to be redox-sensitive: the IL-1 receptor-associated kinase (IRAK), involved in the early events of IL-1–induced activation of NF-κB (10). IL-1–induced IRAK kinase activity is inhibited by several thiol-oxidizing or modulating agents including PDTC. The inhibitory effect of PDTC was reversible, provided the GSH content was high. This suggests that the effect of PDTC was glutathione-dependent (46). At first glance, these results are intriguing as antioxidants are generally described to inhibit IL-1–induced cell activation, but a recent article showed that IRAK kinase activity is not required for IL-1–induced NF-κB activation: IRAK could function as an "adaptor" between IL-1 receptor and the downstream activation cascade, rather than a kinase (49).

Although it is generally agreed that NF-κB activation is redox-regulated, the following three reasons indicate that such regulation does not apply under all circumstances. First, the redox regulation of NF-κB strongly depends on the cell redox status and the culture conditions (12), as well as on the cell type considered (7). Second, several molecules largely used to inhibit NF-κB, like PDTC, were initially considered as antioxidant molecules. It then appeared that they can also exert oxidant properties. PDTC, and dithiocarbamates in general are metal chelators, radical scavengers, and oxidizers of intracellular thiols, including glutathione (for a review, see 31). Third, most studies on NF-κB activation were initially based on gel shifts realized with reducing agents, thereby masking a possible inhibitory effect on the DNA binding capacity of NF-κB (8, 48). In our cell system, PDTC inhibited IL-1–induced DNA-binding of NF-κB and NF-κB-dependent

TABLE 2. EFFECTS OF VARIOUS ANTIOXIDANT MOLECULES AND OF *N*-ETHYL MALEIMIDE ON IL-1-INDUCED NF-κB ACTIVATION, MEASURED AT THE LEVEL OF IκB DEGRADATION, NF-κB DNA-BINDING CAPACITY, AND IL-6 SYNTHESIS

	Inhibition of IL-1-induced NF-κB activation		
	IκB degradation (western blot)	*DNA binding of NF-κB (gel shift)*	*Transcription activity (IL-6 synthesis)*
PDTC (1–100 μM)	No effect	Inhibition (66%)	Inhibition (76%)
Curcumin (1–100 μM)	Inhibition (100%)	Inhibition (100%)	Inhibition (94%)
Taxifolin (50–250 μM)	No effect	No effect	Inhibition (92%)
Trolox C (1–100 μM)	No effect	No effect	No effect
Ergothioneine (10–100 μM)	No effect	No effect	No effect
N-Ethylmaleimide (1–5 μM)	No effect	Inhibition (33%)	Inhibition (78%)

Each molecule was tested at various concentrations (indicated in parentheses). The highest concentration of the molecule and the corresponding percentage of inhibition compared with those in cells stimulated with IL-1 alone are noted whenever inhibition is observed.

transcription, but not IκB degradation, suggesting that the inhibitory effect of PDTC occurs in the nucleus. Nevertheless, this is not always the case as TNFα-induced IκB degradation is inhibited by PDTC in transformed endothelial cells (7).

In contrast to PDTC, curcumin inhibited not only NF-κB DNA-binding activity and IL-6 expression, but also IL-1–induced IκB degradation, indicating that the inhibitory effect of curcumin occurred at the cytoplasmic level of the NF-κB activation process. This inhibitory effect of curcumin could be attributed to the well known antioxidant properties of the molecule (25), however, curcumin alone induced some oxidation of intracellular glutathione (Fig. 8). This unexpected result is not due to direct oxidation of GSH or to direct inhibition of glutathione reductase (data not shown). Currently, no molecular mechanism can explain curcumin-induced oxidation of glutathione, but this phenomenon can explain the results of Kuo and co-workers, who showed that the curcumin-induced apoptosis in promyelocytic leukemia HL-60 cells was inhibited by antioxidants (26). Once again, it shows that a molecule considered as an antioxidant in a particular system may behave as an oxidant in another system. However, the inhibitory effect of curcumin on NF-κB activation is not necessarily linked to the oxidant/antioxidant properties of the molecule, as curcumin also inhibits IL-1–induced IKK activity, but fails to prevent IKK-mediated IκBα degradation (22), suggesting that curcumin inhibits an upstream signal in the transduction cascade of IL-1.

The other antioxidant molecules tested in our system, taxifolin, Trolox C, and ergothioneine, inhibited neither IL-1–induced IκB degradation nor DNA-binding capacity of NF-κB (see Table 2). Only taxifolin inhibited IL-6 synthesis, but this was probably due to the effect of another transcription factor regulating IL-6 gene expression as NF-κB DNA-binding capacity was not affected by taxifolin. Interestingly, although vitamin E derivatives like Trolox C have been shown to inhibit NF-κB-dependent gene transcription in some cell types (45), this molecule had no effect on NF-κB activation in other cell systems (13).

Taken together, these data indicate that the conclusions based on the use of so-called antioxidant molecules in the field of cytokine-induced NF-κB activation should be assessed with caution. There are many steps in cell activation induced by a cytokine like IL-1 on which they can act. Their effect may also vary if they are used *in vitro* or in cell culture, and the culture medium, the cell type, and the extraction procedure must also be considered.

That IL-1 activation leads to intracellular glutathione oxidation was also demonstrated. This does not, however, mean that GSSG is necessary for NF-κB activation. Indeed, our observation that some antioxidant molecules have no influence on NF-κB activation suggests that the prooxidant status of the cytoplasm is not a prerequisite for NF-κB activation under all circumstances.

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ABBREVIATIONS

GSH: reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; IKK, IκB kinase; IL-1, interleukin-1; IL-6, interleukin 6; IRAK, interleukin-1 receptor-activated kinase; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; TNFα, tumor necrosis factor-α.

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