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The relationship between the anti-inflammatory effects of curcumin and cellular glutathione content in myelomonocytic cells

Eva-Maria Strasser *, Barbara Wessner, Nicole Manhart, Erich Roth

Department of Surgery, Research Laboratories, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

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Abstract

Oxidative stress plays an important role during inflammatory diseases and recent therapies have focused on antioxidant administration to diminish oxidative stress and to arrest inflammatory processes. In this study, we investigated the impact of the GSH modulating effects of curcumin, a naturally derived polyphenol, on inflammatory processes in myelomonocytic U937 cells.

One hour after administration of 10 μmol/l curcumin reactive oxygen species (ROS) production was significantly increased in undifferentiated U937 cells (+43%). Twenty-four hour after addition of curcumin, a significantly decreased ROS concentration was found (−32%), whereas GSH (+110%) and GSSG (+88%) content increased. A higher concentration of curcumin (25 μmol/l) caused an even stronger increase of GSH (+145%) and GSSG (+101%), but significantly decreased percentage of living cells to 84%.

The increased GSH content of differentiated U937 cells after pre-incubation with curcumin was associated with lowered ROS production, nuclear factor kappa B (NFκB) activation (−34%) and tumor necrosis factor alpha (TNF-α) secretion (−51%) after LPS exposure. Curcumin inhibited TNF-α formation was also seen after GSH depletion by buthionine sulfoximine (BSO).

This study shows that the antioxidative effects of curcumin are preceded by an oxidative stimulus, which is time and dose-dependent. Excessive concentrations of curcumin may even harm cells, as cell viability was decreased, in spite of elevated GSH contents. There was no clear relationship between intracellular GSH concentrations and the anti-inflammatory effects of curcumin.

Keywords: Curcumin; Antioxidative; Prooxidative; Anti-inflammatory; Glutathione; Reactive oxygen species

1. Introduction

There is increasing evidence that oxidative stress plays a crucial role during development and progression of inflammatory diseases. The most important intracellular antioxidative defense against oxidative stress is the tripeptide glutathione (GSH), which is oxidized to glutathione disulfide (GSSG) while it scavenges free radicals. During inflammatory diseases reduced levels of GSH and/or increased levels of free radicals are detected [1,2], indicating the involvement of oxidative stress. Recent publications have shown that an oxidized cytosolic environment amplifies activation of nuclear factor kappa B (NFκB), which plays a critical role during inflammatory processes by activating many genes encoding for proinflammatory cytokines and immunoregulatory mediators [3]. Some publications have also suggested that reactive oxygen species (ROS) influence inflammatory processes through mechanisms that are dependent on NFκB [4]. Rose et al. have shown that the production of tumor necrosis factor alpha (TNF-α) is regulated by a ROS-activated NFκB pathway in Kupffer cells [5]. Additionally, pre-treatment of human bronchial epithelial cells with *N*-acetyl cysteine, a well known antioxidant, decreases TNF-α-induced activation of NFκB and IL-8 promoter-mediated reporter gene expression [6]. Moreover, the impact of GSH on immune function has been investigated and it has been discovered that GSH depletion in rats impairs T-cell and macrophage

Abbreviations: BSO, buthionine sulfoximine; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; GSH, glutathione; GSHt, total glutathione; GSSG, glutathione disulfide; LPS, lipopolysaccharide; MCF, mean channel fluorescence; NFκB, nuclear factor kappa B; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha

* Corresponding author. Tel.: +43 1 40400 6959; fax: +43 1 40400 6782.
E-mail address: eva.strasser@meduniwien.ac.at (E.-M. Strasser).

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immune function [7]. In this respect, recent investigations for treatment of inflammatory diseases have focused on antioxidant administration to diminish oxidative stress and thus arrest inflammatory processes. Therefore, discovery and investigation of new substances with antioxidative capacities are of particular clinical interest.

Curcumin (diferuloylmethane) is a low molecular weight polyphenol and the main component of the rhizomes of the plant *Curcuma longa* L. Most experimental studies of this substance focus on its cancer chemopreventive effects and it has been demonstrated that curcumin inhibits tumor initiation and promotion in various tissues during animal studies [8,9]. In this respect, some clinical studies have been published recently [10,11]. Several other experimental studies have revealed that curcumin is a potent antioxidant, as it is able to scavenge ROS [12,13], increase GSH content [14,15] and decrease lipid peroxidation [16]. These antioxidative effects of curcumin might be useful for the prevention or treatment of inflammatory processes.

This study was therefore designed to investigate the impact of curcumin-evoked increase in GSH on the occurrence of ROS and inflammatory reactions in myelomonocytic cells. Interestingly, our results revealed that at least TNF-α formation was not dependent on cellular GSH content.

2. Materials and methods

2.1. Chemicals

All chemicals were derived from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated and were of the highest grade obtainable. Curcumin was dissolved in DMSO so that the final concentration of DMSO in the cell culture medium never exceeded 0.1%. All substance stock solutions were prepared freshly before use.

2.2. Cell culture

The human myelomonocytic cell line U937 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were maintained in continuous cell suspension at 37 °C under 5% CO_2 and humidified air in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mmol/l glutamine (Gibco, Paisley, UK) and 10% fetal bovine serum (FBS; Linaris, Bettingen, Germany).

2.3. Treatment of undifferentiated U937 cells

Undifferentiated U937 cells were used to study the antioxidative effects of curcumin. In former studies we found that GSH content of U937 cells reached a maximum after 24 h cultivation at standard conditions [17]. Therefore, 0.2×10^6 cells/ml were incubated in RPMI medium containing curcumin (1–25 μmol/l) for 24 h at standard conditions. Respective controls were treated with an equal volume of DMSO. After incubation cell counts of living and dead cells were examined by trypan blue staining and cell viability was calculated. GSH and GSSG levels of the cells were assessed after 24 h incubation with increasing concentrations of curcumin (1–25 μmol/l). The production of ROS was measured 1, 3, 6, 12 and 24 h after addition of 10 or 25 μmol/l curcumin.

2.4. Differentiation of U937 cells with PMA

As undifferentiated U937 cells fail to produce TNF-α upon lipopolysaccharide (LPS) stimulation (data not shown), we used a 24 h incubation period with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) to differentiate cells and sensitize them to LPS, according to Refs. [18,19]. To investigate influence of PMA on differentiation of the cells, expression of surface antigens (CD11b, CD14) was quantified by flow cytometry using a direct immunofluorescence staining technique. Nondifferentiated (=control) and PMA-differentiated U937 cells were collected with HBSS containing 0.1% sodium azide and 1% FBS, washed and counted. Aliquots (20 μl) of antibodies (CD11b-FITC and CD14-FITC, Immunotech, Marseille, France) were added to stain the cells. After 30 min of incubation at 4 °C, cells were washed twice, resuspended in 500 μl HBSS and analyzed by flow cytometry (XL-EPICS, Beckman Coulter, Fullerton, CA).

2.5. Treatment of PMA-differentiated U937 cells

After 24 h incubation with PMA, cells were pelleted by centrifugation and PMA-containing medium was removed. To investigate the influence of GSH modulation on inflammatory processes fresh medium containing 10 μmol/l curcumin, 150 μmol/l buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, or curcumin (10 μmol/l) + BSO (150 μmol/l) was added and cells were again incubated for 24 h. After that, on one hand GSH and GSSG levels were measured. On the other hand 1 μg/ml LPS (serotype: O111:B4) [18] was added to pre-treated cells. ROS formation was assessed before as well as 3 and 6 h after addition of LPS. Activation of NFκB was examined 30 min after LPS addition, as former experiments revealed the strongest activation at this time point (data not shown). TNF-α production was assessed 6 h after LPS administration.

2.6. Determination of GSH and GSSG

Levels of GSH and GSSG were determined using the Calbiochem® GSH/GSSG Ratio Assay Kit (Calbiochem, San Diego, CA), based on the method originally described by Tietze [20]. Briefly, $(5–10) \times 10^6$ cells were harvested

after the respective treatment, washed twice and resuspended in cold PBS. Cell suspension was divided for total GSH (GSHt) and GSSG samples. For GSSG analysis, the GSH scavenger 1-methyl-2-vinylpyridinium trifluoromethane sulfonate was added. GSH_t- and GSSG-samples were snap frozen and stored at −80 °C until further analysis. After thawing, samples were processed according to the manufacturer's instructions. The assay mixture consisted of samples, blank or standards, 5,5′-dithiobis-(2-nitrobenzoic acid) as chromogen and GSSG reductase. The reaction was started by adding NADPH. The absorbance was followed at 412 nm for 3 min on a U-3000 spectrophotometer (Hitachi High Technologies, Finchampstead, UK). After determination of reaction rates, GSH_t and GSSG-contents were calculated by comparing the obtained reaction rates with those of the provided standards. GSH concentrations were calculated by subtracting 2 GSSG- from GSH_t-contents.

2.7. Measurement of reactive oxygen species

The production of ROS was measured using 6-carboxy-2′,7′-dichlorodihydrofluorescein-diacetate, di(acetoxymethylester) (c-H_2DCFDA; Molecular Probes, Eugene, OR) as indicator, which forms fluorescent carboxy-dichlorofluorescein upon oxidation by intracellular ROS. As curcumin is known to possess fluorescent properties, all our ROS experiments were corrected with the appropriate control sample containing only curcumin. Briefly, treated cells were washed twice and counted. Cells (0.5×10^6) were loaded with 5 μmol/l c-H_2DCFDA and incubated at 37 °C for 1.5 h. After washing cells twice with cold PBS, fluorescence emission from carboxy-dichlorofluorescein was detected by flow cytometry (XL-EPICS, Beckman Coulter, Fullerton, CA) at a wavelength of 530 ± 30 nm. Typically forward and side scatter signals were used to gate living cells and 10,000 events were measured using linear amplification. Values are expressed as mean channel fluorescence (MCF).

2.8. Measurement of NFκB activation after LPS stimulation

Activation of NFκB was assessed according to the publication by Lewis and Konradi [21] with slight modifications. The whole preparation was performed on ice. Briefly, cultured cells were collected, washed once with ice cold PBS and resuspended in Buffer A (10 mmol/l HEPES, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, H_2O; 0.5 mmol/l phenylmethylsulfonylfluoride (PMSF), 0.5 mmol/l dithiothreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were allowed to swell on ice for 15 min. Lysis of the plasma membrane was achieved by the addition of 2.5% Nonidet P-40. Nuclei were immediately pelleted by centrifugation. Nuclei pellets were resuspended in Buffer C (20 mmol/l HEPES, 0.45 mol/l NaCl, H_2O, 1 mmol/l EDTA; 0.5 mmol/l PMSF, 0.5 mmol/l DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and incubated for 15 min on ice. After centrifugation supernatants were diluted 1:1 in Buffer D (20 mmol/l HEPES, 0.1 mol/l KCl, 0.2 mmol/l EDTA, glycerol 20%; 0.5 mmol/l DTT) and stored at −80 °C until further analysis. A small aliquot was used to determine protein amount according to the method of Bradford [22]. For electrophoretic mobility shift assay (EMSA), 8 μg nuclear proteins were incubated with 1 μg polydeoxy (Inosinate-Cytidylate) Acid (USB Corporation, Cleveland, OH) and a ^{32}P-labeled DNA probe (7500 cpm) with the consensus sequence for NFκB (Promega, Madison, WI). Mixtures were separated on a 4.5% polyacrylamide gel. Quantification of radioactive bands in dried gels was performed on a Molecular Imager FX Pro Plus Phosphor Imager using the Quantity One software Version 4.2.2 (BioRad, Hercules, CA).

2.9. Measurement of TNF-α production after LPS stimulation

TNF-α production was assessed 6 h after LPS addition (1 μg/ml). Cell suspensions were centrifuged to obtain cell-free supernatants, which were stored at −80 °C until measurement. Amount of TNF-α in the supernatants was measured with the semi-automated chemoluminescent immunoassay analyzer Immulite (DPC, Los Angeles, CA).

2.10. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was applied to the raw data by using the Student's *t*-test. In the case of multiple comparisons, one-way analysis of variance (ANOVA) with Tukey post-test was applied using the SPSS software package (SPSS for Windows, Release 8.0.0, Chicago, IL). Probability of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. GSH- and GSSG-modulating effects of curcumin

Undifferentiated U937 cells were exposed to varying concentrations of curcumin (1–25 μmol/l) for 24 h, and intracellular GSH and GSSG amount as well as cell viability were assessed. No significant influence on GSH or GSSG content was found after incubation with 1 μmol/l curcumin. Fig. 1A demonstrates that treatment of the cells with 10 and 25 μmol/l curcumin enhanced GSH content to 211 ± 53% and 245 ± 38%, respectively. GSSG levels were also significantly increased to 188 ± 64% and 201 ± 15%, respectively (Fig. 1B). Due to the fact that GSH and GSSG amount of the cells increased to the same extent, the evaluation of GSH/GSSG ratio indicated no significant change (Fig. 1C). Assessment of cell counts

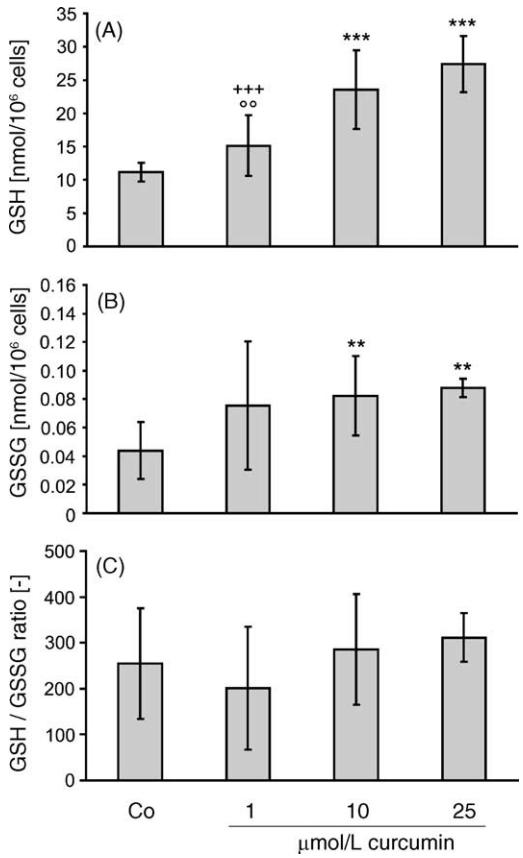

Fig. 1. GSH and GSSG contents modulating effects of curcumin. U937 cells were incubated with increasing concentrations of curcumin (1–25 μmol/l) or vehicle (DMSO) for 24 h. GSH and GSSG content were measured enzymatically. (A) Represents mean GSH amount, expressed as nmol/10^6 cells ± S.D. (B) Represents accompanied mean GSSG amount in nmol/10^6 cells ± S.D. (C) Represents mean GSH/GSSG ratio. Results are calculated from five independent experiments. $^{***}p < 0.001$ vs. GSH content of control cells; $^{\circ\circ}p < 0.01$ vs. GSH amount of cells treated with 10 μmol/l curcumin; $^{+++}p < 0.001$ vs. GSH content of cells treated with 25 μmol/l curcumin; $^{**}p < 0.01$ vs. GSSG content of control cells.

revealed that after 24 h incubation with 10 and 25 μmol/l, cell count of living cells was significantly reduced from $0.31 \pm 0.07 \times 10^6$ cells/ml of control to $0.19 \pm 0.05 \times 10^6$ cells/ml ($p < 0.001$) and $0.14 \pm 0.04 \times 10^6$ cells/ml ($p < 0.001$), respectively, but only the highest concentration significantly increased the number of dead cells at the same time, from $0.017 \pm 0.01 \times 10^6$ cells/ml of control to $0.025 \pm 0.01 \times 10^6$ cells/ml ($p < 0.05$). Whereas cell viability was not affected by treatment of 10 μmol/l curcumin (93 ± 2.8%), 25 μmol/l curcumin significantly decreased cell viability from 95 ± 3.2% of control to 84 ± 6.2% ($p < 0.001$ versus viability of control and cells treated with 10 μmol/l curcumin).

3.2. Biphasic effects of curcumin on ROS production

As an increase of GSSG amount indicated an involvement of oxidative stress, ROS production was measured by flow cytometry 1, 3, 6, 12 and 24 h after addition of curcumin. ROS production was enhanced from 49 ± 5.8 MCF of control to 70 ± 1.7 MCF ($p < 0.01$) and 78 ± 4.1 MCF ($p < 0.01$) after 1 h incubation with 10 and 25 μmol/l curcumin, respectively. However, this increase was followed by a time-dependent decrease of ROS production in the treatment groups. Finally, 24 h after addition of 10 and 25 μmol/l curcumin, ROS levels dropped below the production level of control cells, to 24 ± 2.8 MCF ($p < 0.05$) and 19 ± 5.9 MCF ($p < 0.01$), respectively (Fig. 2).

3.3. Effects of GSH on inflammatory parameters

PMA treatment for 24 h increased expression of CD11b and CD14 by 46 ± 2.8% ($p < 0.05$) and 40 ± 22% ($p < 0.05$), respectively. To investigate the influence of GSH-modulating effects of curcumin on inflammatory parameters (TNF-α, NFκB activation), PMA-differentiated U937 cells were incubated with curcumin, BSO or curcumin + BSO for 24 h prior to LPS stimulation.

3.4. GSH and GSSG content of PMA-differentiated cells

Twenty-four hour incubation of PMA-differentiated cells with 10 μmol/l curcumin did not alter cell viability and increased GSH and GSSG content significantly to 197 ± 35% and 353 ± 162%, respectively. Incubation of PMA-differentiated cells with BSO and curcumin + BSO for 24 h reduced GSH levels to 25 ± 8.3% and 23 ± 6.1%, respectively. GSSG levels decreased in BSO treated cells to 49.0 ± 23.6% and in curcumin + BSO treated cells to 73.8 ± 13.8% (Fig. 3). BSO treatment did not change cell viability.

3.5. ROS production before and after LPS stimulation

PMA-differentiated cells were incubated with curcumin, BSO or curcumin + BSO for 24 h and ROS production was examined before (0 h) as well as 3 and 6 h after addition of LPS. Similar to our results of undifferentiated U937 cells,

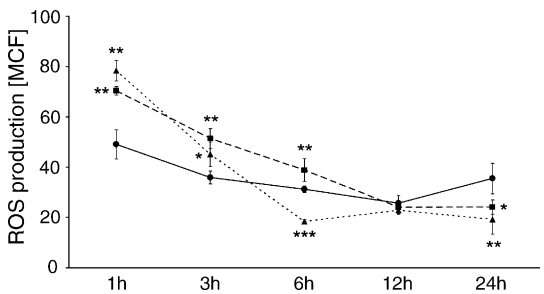

Fig. 2. Biphasic effects of curcumin on ROS production. U937 cells were incubated with 10 μmol/l (■) and 25 μmol/l (▲) curcumin or vehicle (●) for 1, 3, 6, 12 and 24 h. ROS formation was assessed by staining with C-DCF and flow cytometry. Values are mean ± S.D. calculated from five independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. ROS production of control cells at respective time point.

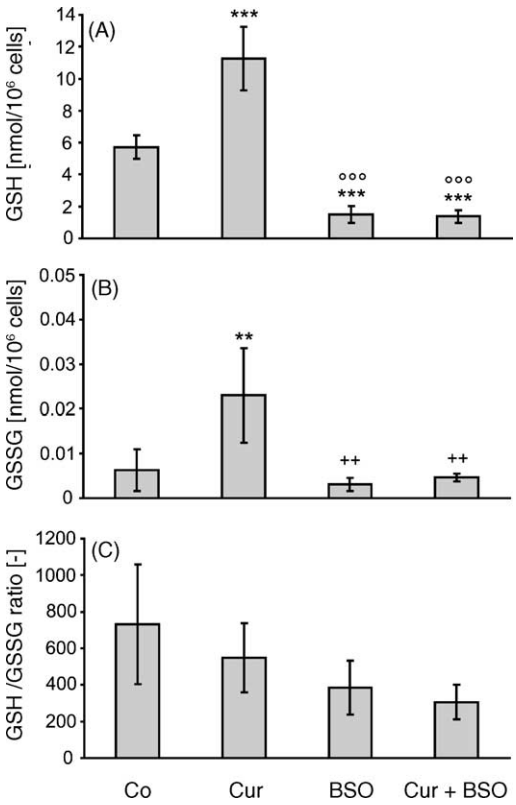

Fig. 3. GSH and GSSG content of PMA differentiated cells. PMA differentiated cells were incubated with vehicle (DMSO), 10 μmol/l curcumin, 150 μmol/l BSO or curcumin + BSO for 24 h. GSH and GSSG content were measured enzymatically. (A) Represents mean GSH amount, expressed as nmol/10^6 cells ± S.D. (B) Represents accompanied mean GSSG amount in nmol/10^6 cells ± S.D. (C) Represents mean GSH/GSSG ratio. Results are calculated from five independent experiments. $^{***}p < 0.001$ vs. GSH content of control cells; $^{\circ\circ\circ}p < 0.001$ vs. GSH content of cells treated with 10 μmol/l curcumin; $^{**}p < 0.01$ vs. GSSG content of control cells; $^{++}p < 0.01$ vs. GSSG content of cells treated with 10 μmol/l curcumin.

24 h incubation of differentiated U937 cells with curcumin resulted in a significant lower ROS production at baseline (0 h) compared to control cells. Administration of LPS significantly increased ROS production in every treatment group 3 h thereafter. On the other hand, 3 and 6 h after LPS addition the amount of ROS was significantly lower in curcumin pre-treated cells compared to control at the corresponding time point. After 6 h ROS amount of curcumin pre-treated cells returned to initial values. BSO pre-treatment caused the highest ROS amount after 3 h and even 6 h after LPS supply, ROS levels were still significantly higher than control levels. Six hours after LPS addition, ROS production of curcumin + BSO pre-treated cells was significantly lower than ROS formation of BSO treated cells (Table 1).

Table 1
Influence of LPS on ROS production

	Control (MCF)	Curcumin (MCF)	BSO (MCF)	Curcumin + BSO (MCF)
0 h	27.1 ± 3.8	19.2 ± 2.8$^{\circ\circ}$	31.2 ± 2.0	31.0 ± 4.1
3 h	37.9 ± 6.5*	29.2 ± 3.4$^{***,\circ}$	44.1 ± 4.9$^{***,\circ}$	41.0 ± 2.9**
6 h	32.6 ± 3.2*	20.4 ± 3.4$^{\circ\circ\circ}$	38.2 ± 1.7$^{***,\circ}$	31.5 ± 3.1^{++}

PMA-differentiated cells were incubated with 10 μmol/l curcumin, 150 μmol/l BSO, curcumin + BSO or vehicle for 24 h. ROS formation was measured before (0 h) as well as 3 h and 6 h after addition of 1 μg/ml LPS, by flow cytometry. Values are mean ± S.D., calculated from five independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ and *** $p < 0.001$ vs. ROS amount of vehicle, curcumin, BSO or curcumin + BSO treated cells at baseline (0 h). $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.01$ vs. ROS amount of control cells at respective time point. $++p < 0.01$ vs. ROS amount of BSO treated cells (6 h).

3.6. NFκB activation after LPS stimulation

NFκB activation of PMA-differentiated and curcumin, BSO, curcumin + BSO or vehicle pre-treated cells was assessed 30 min after LPS addition. LPS administration led to a significant increase in NFκB activation compared to untreated control cells. Supershift analysis revealed that this NFκB protein was composed of p65 and p50. Curcumin pre-treatment was able to diminish NFκB activation significantly (−34 ± 16%). Whereas no significant change of NFκB activation was detected in BSO and curcumin + BSO groups, supershift analysis revealed that in these groups NFκB proteins contained no p50 (Fig. 4).

3.7. TNF-α production after LPS stimulation

LPS stimulation for 6 h increased TNF-α production from 9.4 ± 1.2 to 236 ± 28 pg/ml in vehicle pre-treated cells. Pre-incubation of the cells with curcumin was able to reduce cytokine production to 114 ± 28 pg/ml (−51%; $p < 0.001$). BSO treatment elevated TNF-α production

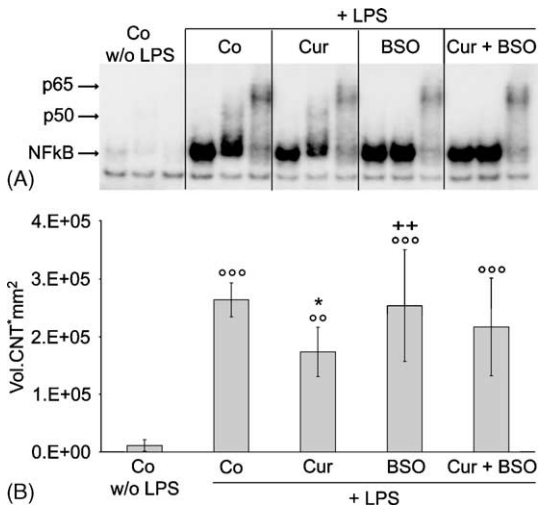

Fig. 4. NFκB activation after LPS stimulation. PMA-differentiated cells were incubated with 10 μmol/l curcumin, 150 μmol/l BSO, curcumin + BSO or vehicle (DMSO) for 24 h. Nuclear extracts of samples were subjected to electrophoretic mobility shift assay (EMSA) for NFκB DNA-binding activity 30 min after LPS addition. One sample was not treated with LPS as control (Co w/o LPS). Supershift analysis of p65 and p50 proteins was performed. (A) Shows one representative EMSA out of three independent experiments. (B) Represents EMSA results of three independent experiments as mean ± S.D. $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.001$ vs. NFκB activity of Co w/o LPS; $^{*}p < 0.05$ vs. NFκB activity of control cells; $^{++}p < 0.01$ vs. NFκB activity of curcumin pre-treated cells.

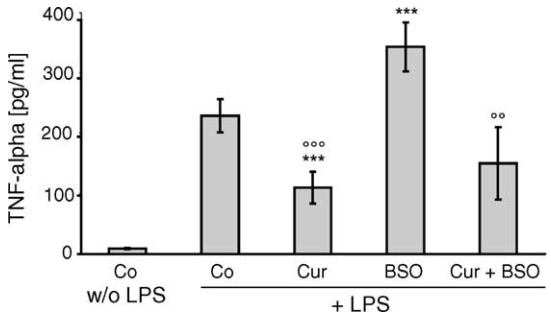

Fig. 5. TNF-α production after LPS stimulation. PMA-differentiated cells were incubated with vehicle (DMSO), 10 μmol/l curcumin, 150 μmol/l BSO or curcumin + BSO for 24 h. Cell-free supernatants were obtained 6 h after addition of 1 μg/ml LPS. One sample was not treated with LPS as control (Co w/o LPS). Amount of TNF-α in the supernatants were measured with the semi-automated chemoluminescent immunoassay analyzer Immulite and expressed as pg/ml. Values are expressed as mean ± S.D., calculated from five independent experiments. $^{***}p < 0.001$ vs. LPS-treated control cells; $^{°°}p < 0.01$ and $^{°°°}p < 0.001$ vs. BSO group.

further to 354 ± 42 pg/ml (+50%, $p < 0.001$). Curcumin + BSO treated cells showed TNF-α production levels of 155 ± 62 pg/ml (n.s. versus LPS treated control, $p < 0.001$ versus BSO) (Fig. 5).

4. Discussion

The present study revealed that curcumin exerts a time-dependent oxidative–antioxidative reaction pattern on myelomonocytic cells. One hour after addition of curcumin the production of ROS was induced. At a later time point ROS levels of curcumin pre-treated cells were significantly lower than control levels. These results were accompanied by increased GSH and GSSG contents. Our data therefore clearly revealed that early after the supply, curcumin provokes oxidative stress, which seems to be gradually compensated by stimulation of the GSH-GSSG cycle. Although we did not quantify GSH synthesis in this study, Piper et al. support this hypothesis, showing that curcumin stimulates γ-glutamate-cysteine ligase activity when rats were fed with increasing dosages of curcumin (75–500 mg/(kg d)) for 14 days [23].

Several authors have already investigated the effect of curcumin on GSH content and found an increase of total GSH after curcumin administration [24,25]. However, most of these studies did not measure the oxidized form of this redox couple, GSSG. Our study revealed that curcumin increased GSH but also GSSG, resulting in an unchanged redox potential, characterized by the ratio GSH/GSSG.

The hypothesis that polyphenols, besides their antioxidative effects, also have prooxidative properties, is nothing new, but often forgotten. After many publications have shown tremendous antioxidative and beneficial effects of polyphenols, now reports steadily appear about apparently contradictory investigations of the same substances, which might be related to their prooxidative effects. One example is a recent publication by Surayanarayana et al. showing that dietary supplementation of 0.002% curcumin reduced the onset and maturation of cataract in diabetic rats, but higher concentrations of curcumin (0.01%) in the diet accelerate the maturation of cataract [26]. Moreover, Galati and O'Brien recently reviewed investigations about the prooxidative related toxicity of dietary phenolics. Almost every well known polyphenolic antioxidant was found to have prooxidative potential, including resveratrol, green/black tea phenolics, quercetin, capsaicin, curcumin and many more. They conclude that every phenol ring-containing flavonoid yields cytotoxic phenoxyl radicals upon oxidation by peroxidases [27]. It is also interesting that in our study a concentration of 25 μmol/l curcumin, which caused the highest GSH amount, also reduced cell viability. Unpublished observations revealed this cell viability change was due to increased apoptosis and cell cycle arrest, which might be related to excessive oxidative effects. In this respect, it is worth mentioning that most of the in vitro studies dealing with curcumin as an antioxidant, use concentrations as high as 100 μmol/l. Although in these studies decreased ROS formation is observed [28,29] after 1–2 h incubation with curcumin, it can be assumed that these concentrations may most likely cause oxidative cell damage at a later time point.

During inflammatory processes, the activation of NFκB plays a crucial role, as it is responsible for the activation of inflammatory genes, which encode for cytokine production. Although the exact regulatory mechanisms of NFκB activation and inhibition are still unknown, there is no doubt that the redox potential has a strong impact on it. It has been shown that cytosolic release of its inhibitor IκB and subsequent NFκB nuclear translocation is induced by ROS [30]. By contrast, antioxidants, which reduce cytosolic redox potential, are able to diminish NFκB activation [31]. Although it has been shown earlier that curcumin inhibits NFκB activation and cytokine production [32,33], until to date there are no publications investigating whether anti-inflammatory activities of curcumin are related to its GSH-enhancing properties. For this reason we established a model system of PMA-differentiated cells which respond with increased ROS production, NFκB activation and TNF-α secretion upon LPS stimulation. Interestingly, PMA-differentiated cells showed decreased GSH and GSSG content compared to control cells. In this respect, Kirlin et al. have investigated the change of GSH and GSSG during sodium butyrate induced differentiation of HT29 cells. As a time-related decrease in GSH, increase in GSSG and decrease of γ-glutamate-cysteine ligase was found during differentiation, they concluded that changes in redox state could regulate transcriptional activation of redox sensitive genes [34].

Concerning our results of the early prooxidative effects we used a 24 h incubation period with curcumin prior to LPS stimulation to increase GSH content. This modulation of GSH with curcumin resulted in a significantly decreased

NFκB activation and TNF-α production, accompanied by significantly lower ROS production. On the other hand inhibition of the GSH synthesis by BSO increased TNF-α production and ROS formation. However, in spite of inhibited GSH synthesis the administration of curcumin + BSO reduced TNF-α formation as well, compared to BSO treated cells. In this respect, it is interesting that although the cytokine production was significantly different between BSO and curcumin + BSO groups, we detected no change in NFκB activation 30 min after LPS addition between these groups. One explanation for this finding might be that beside NFκB other transcription factors are involved in TNF-α production (e.g., AP-1). Duvoix et al. have shown that curcumin effectively inhibited tumour necrosis factor-alpha- and phorbol ester-induced binding of AP-1 and NFκB transcription factors to sites located on the GSTP1-1 gene promoter [35], while Kamata et al. have shown that activation of AP-1 is significantly enhanced by BSO in PC-12 cells [36]. Therefore, it would be interesting to investigate whether in this experimental cell system other transcription factors have more impact on TNF-alpha production than NFκB.

Concerning the discrepancy of ROS production between BSO and curcumin + BSO pre-treated cells, some publications have stated that other signaling pathways departing from GSH may influence cytokine production. In this respect, it has already been shown that in human T lymphocytes a longitudinal exposure to chronic low levels of oxidative stress causes a decrease in the DNA-binding activity of transcription factors that regulate cytokine genes via suppression of mitogen induced transmembrane signaling (protein tyrosine phosphorylation and calcium mobilization) [37]. According to this publication it would be interesting to investigate whether the oxidative stimulus caused by curcumin leads to receptor and signal cascade variation, thereby making the cells less reactive to LPS exposure.

Overall we conclude that the antioxidative effects of curcumin are preceded by an oxidative stimulus, whereby this effect seems to be dose- and time-dependent. Interestingly, TNF-α release after LPS stimulation seems rather to depend on the actual amount of ROS than on the initial GSH content.

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