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Methylglyoxal suppresses TNF-α-induced NF-κB activation by inhibiting NF-κB DNA-binding

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ABSTRACT

Methylglyoxal is a cytotoxic metabolite that is produced *in vivo* mainly from glycolysis. Increased production of methylglyoxal can be induced by tumor necrosis factor and occurs in a number of pathological conditions, including diabetes and neurodegenerative disorders. Methylglyoxal is highly reactive and can modify proteins, which results in the formation of advanced glycation end products. Yet, we, and others, have recently proposed a role for methylglyoxal as a signaling molecule. In this study, we show that methylglyoxal inhibits TNF-induced NF-κB activation and NF-κB-dependent reporter gene expression by inhibiting the DNA binding capacity of NF-κB p65. Methylglyoxal slightly delayed, but did not inhibit, TNF-induced degradation of IκBα and strongly inhibited TNF-induced NF-κB-dependent re-synthesis of IκBα. The TNF-induced nuclear translocation of NF-κB p65 was also delayed, but not inhibited, in the presence of methylglyoxal. TNF-induced phosphorylation of p65 was not affected by methylglyoxal. We show that the conserved Cys 38 residue, which is located in the DNA binding loop of NF-κB p65 and responsible for the redox regulation of the transcription factor, is involved in the methylglyoxal-mediated inhibition of p65 DNA-binding. Furthermore, overexpression of p65 inhibited TNF-induced cell death; however, in the presence of exogenously added methylglyoxal, overexpression of p65 caused far greater TNF-induced cell death. These findings suggest that methylglyoxal provides another control mechanism for modulating the expression of NF-κB-responsive genes and that methylglyoxal may be responsible for tipping the balance towards TNF-induced cell death in cells with constitutive NF-κB activation.

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Abbreviations: MG, methylglyoxal; GLO1, glyoxalase I; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; AGEs, advanced glycation end products; NLS, nuclear localization signal

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1. Introduction

Methylglyoxal (MG) is a highly reactive and cytotoxic metabolite that is primarily produced during normal cellular metabolism through the elimination of phosphate from dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate, both intermediates of glycolysis. MG can also be synthesized by MG synthase [1]. MG is normally detoxified to D-lactate by the glyoxalase system, which is comprised of glyoxalase I (GLO1), and II (reviewed in [2]). However, the full biological function of the glyoxalase pathway has not yet been elucidated. The work of Szent-Györgyi suggested that GLO1 and its substrate MG were involved in the regulation of cell division, but a direct mechanistic link has yet to be identified [2]. For many years, MG has been known to be carcinostatic, but its direct use as an anticancer drug has been prevented by its rapid detoxification *in vivo* by the glyoxalase system. This characteristic provided the rationale for the development of GLO1 inhibitors as potential anti-cancer agents [3,4].

Increased expression of GLO1 is associated with several diseases, including diabetes, Alzheimer's disease [5] and several types of cancer [6–8]. GLO1 is particularly overexpressed in the more aggressive and invasive forms of ovarian cancer [9] and the Her-2/*neu*-positive breast cancers, which are refractory to various types of therapy and associated with a poor prognosis [10]. Furthermore, over expression of GLO1 is involved in the resistance of human leukemia cells to anti-tumor agent-induced apoptosis [11].

Up to now, why the cytotoxic component MG is produced during normal cellular metabolism has been a mystery. But, we [12] and others [13], have proposed a role for MG as a signaling molecule. Increased levels of methylglyoxal lead to the rapid modification of proteins to generate advanced glycation end products (AGEs). Formation of AGEs contributes to the development of pathological conditions *in vivo*, such as diabetes and cancer [11,14,15].

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that plays a role in the pathophysiology of various diseases (reviewed in [16]). Furthermore, at low concentration, TNF can promote tumor growth [17] and, at high concentration, TNF has potent anti-tumor and anti-malignant cell effects [18]. In various cell types, TNF induces two signaling pathways: one leads to gene activation, which is primarily mediated through activation of NF-κB; and the other leads to cell death by either apoptosis or necrosis [18]. The final outcome of TNF-induced signaling is dependent on the cell type and the cross-talk between the two pathways [19]. For instance, NF-κB can act as an anti-apoptotic transcription factor that leads to the induction of anti-apoptotic proteins.

We have recently described how TNF-induced necrosis – characterized by oxidative stress – in the fibrosarcoma cell-line L929 is accompanied by increased intracellular concentrations of methylglyoxal. This – along with the TNF-induced phosphorylation of GLO1 – leads to MG-modification of specific target molecules (MG-derived AGEs) [12]. Furthermore, exogenously added MG is strongly synergistic with TNF-induced cell death and can even sensitize resistant L929 clones to TNF-induced cell death (our unpublished results). It is well known that NF-κB is necessary and sufficient for the prevention of TNF-induced cell death [20,21]. Therefore, the purpose of this present investigation was to examine whether the synergistic action of MG on TNF-induced cell death is (partially) mediated through an effect on NF-κB.

2. Materials and methods

2.1. Cell lines

L929 cells were cultured in Dulbecco's modified Eagle's medium with glutamax supplemented with heat-inactivated fetal calf serum (10%, v/v), penicillin (100 units/ml), and streptomycin (0.1 mg/ml), at 37 °C in a humidified incubator under an 8% CO_2 atmosphere. Human carcinoma (HeLa) cells were grown in Dulbecco's modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambrex) at 37 °C in a humidified incubator under an 8% CO_2 atmosphere. Human umbilical vein endothelial cells (HUVEC) cells were grown in EGM-2 medium (Cambrex) according to manufacturer's instructions, at 37 °C under a 5% CO_2 humidified atmosphere.

2.2. Reagents

Murine TNF (mTNF) was obtained from Roche (Roche Diagnostics, Mannheim, Germany). Methylglyoxal was obtained from Sigma. Antibodies against phosphorylated IκBα, IκBα, phophorylated p65 and ubiquitin were from Cell Signaling Technology; anti-p65 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-coupled secondary antibodies from Amersham Biosciences; and Alexafluor 488-coupled secondary anti-mouse antibody from Molecular Probes.

2.3. Preparation of cell lysates

L929 cells were seeded 24 h prior to the experiment. After TNF incubations (1000 U/ml), the cells were rinsed three times with ice-cold PBS buffer, and total cell lysates for the NF-κB Transcription Factor Assay were prepared in Totex buffer (20 mM Hepes/KOH pH 7.9; 350 mM NaCl; 20% glycerol; 1% NP-40; 1 mM $MgCl_2$; 0.5 mM EDTA pH 8.0; 0.1 mM EGTA; 5 mM DTT; 10 mM NaF; 40 mM β-glycerophosphate; Protease inhibitor cocktail) [22]. Cell lysates for monitoring the phosphorylation, ubiquitination and protein levels of IκBα and p65 were prepared in a CHAPS-containing cytosol extraction buffer [23].

2.4. Measurement of the NF-κB p65 DNA binding activity

The DNA binding of NF-κB p65 subunit was measured with a colorimetric non-radioactive NF-κB p65 Transcription Factor ELISA Assay (Chemicon® International, CA, USA) according to the manufacturer's instructions.

2.5. NF-κB-dependent reporter gene expression assay

For the reporter assays, we used L929sA cells that are stably transfected with a synthetic reporter construct $(IL6\text{-}\kappa B)_3$-50IL6P-luc + and the selection vector pPGKβGeobpA (encoding a $[neo]^r$-β-galactosidase fusion protein conferring resistance to G418 as well as constitutive β-galactosidase enzymatic

activity). These cells and constructs were described previously by [24]. The $(IL6\text{-}\kappa B)_3$-50IL6P-luc + plasmid contains a concatenated trimer of the IL6-κB motif atgtGGGATTTTCCCatg (capitals indicating the IL6-κB core sequence) in front of a minimal IL6 promoter in the pGL3 reporter vector (Promega Biotec, Madison, WI), which encodes a luciferase reporter gene. Luciferase assays were carried out according to the manufacturers instructions (Promega Biotec). Cells were lysed in a buffer containing 25 mM Tris phosphate (pH 7.8), 2 mM DTT, 2 mM cyclohexanediaminetetraacetic acid, 10% glycerol and 1% Triton X-100 for 15 min at room temperature. From the cell extracts, 20 μl was transferred into a 96-well plate together with 50 μl of the luciferase assay reagent (20 mM Tricine, 1.07 mM $[(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O]$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzymeA, 470 μM D-luciferin, 530 μM ATP). This mixture was then analysed in a luminescence microplate counter (TopCount; Packard, Meriden, Conn.) for luciferase activity and corrected for β-galactosidase activity (GalactoLight kit; Tropix, Bedford, MA).

2.6. Western blotting

Proteins were separated by SDS-PAGE (12%) and transferred to a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the respective antibodies, followed by ECL-based detection (Amersham Pharmacia Biotech).

2.7. Confocal immunofluorescence microscopy

L929 cells were plated on glass coverslips 48 h prior to the experiment. After incubation with TNF (and/or with 750 μMG) for the respective time periods, the cells were washed three times with PBS and then fixed with 100% ice-cold methanol for 10 min at −20 °C. The cells were then rinsed three times with PBS and incubated with a primary monoclonal antibody against p65 NF-κB (1:100) for 1 h at room temperature. The cells were washed three times with PBS and incubated with an anti-mouse Alexa-488-conjugated secondary antibody for 1 h and again washed three times with PBS. The coverslips were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA). Images were taken with a Biorad laser scanning confocal microscope.

2.8. Plasmids

The pcDNA-p65 wild type plasmid was obtained from Dr. R. Hay. This plasmid was used as a template to construct the C38A mutant. The forward 5′-GCGCTTCCGCTACAAGGCC-GAGGGGCGCTCCGCGGG-3′ and reverse 5′-CCCGCGGAGC-GCCCCTCGGCCTTGTAGCGGAAGCGC-3′ primers were used to mutate the Cys38 to an Ala residue using a site-directed mutagenesis kit (Stratagene). The mutation was confirmed by sequencing.

2.9. Transient transfection experiments

Subconfluent monolayers of HeLa cells (6-well plates) were used for transient transfections using the lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Cells were transfected with pcDNA-p65 WT and with pcDNA-p65 C38A mutant. Mock-transfected cells were used as control. 24 h after transfection, the cells were treated for 1 h with 1000 U/ml TNFα and/or methylglyoxal concentrations, as indicated. After treatment, the cells were washed three times with phosphate-buffered saline and lysed on ice in Totex buffer.

2.10. Measurement of TNF-induced cell death by flow cytometry

Cell death in L929-derived cell lines has been described previously [23]. Briefly, Cell death was induced by the addition of TNF (100 U/ml) to the cell suspension. Cell death was measured by quantifying PI-positive cells by FACS (FACS Calibur, Becton Dickinson, San Jose, CA). Routinely, 3000 cells were analysed. Cell death is expressed as the percentage of PI-positive cells in the total cell population.

3. Results

3.1. Methylglyoxal suppresses TNF-α-induced NF-κB activation

To determine whether MG had an effect on TNF-induced NF-κB activation, we measured the DNA binding of the NF-κB p65 subunit. L929 cells were incubated with TNF (1000 U/ml) in the presence of increasing concentrations of MG for 1 h, a time point at which there is no detectable cell death. At the end of the incubation, total cell lysates were prepared in Totex buffer (see Section 2). The DNA binding of the NF-κB p65 subunit was determined by the colorimetric non-radioactive NF-κB p65 Transcription Factor Assay in 96-well format (Chemicon®). In this assay, a double-stranded biotinylated oligonucleotide, containing the consensus sequence for NF-κB binding, is incubated with total cellular extracts. This capture probe binds only the active form of NF-κB p65. The mixture is then incubated on a streptavidin-coated plate. The amount of p65 bound to the oligonucleotide is then detected with a specific polyclonal anti-NF-κB p65 primary antibody and a HRP-conjugated secondary antibody. A representative experiment of three independent experiments is shown in Fig. 1. As is clear from this figure, increasing concentrations of MG strongly inhibit the TNF-induced DNA binding of the NF-κB p65 subunit in L929 cells. Maximum inhibition (80%) of p65 DNA binding is observed at 750 μM MG. The rather modest induction of NF-κB activation by TNF alone is due to the time point chosen, namely 1 h of TNF treatment. This is a time point when there is already a considerable TNF-induced NF-κB-dependent re-synthesis of the NF-κB inhibitor IκBα (see Fig. 3). Analysis of the protein levels of p65 by Western blotting showed that they are not affected by MG (data not shown). This excluded the possibility that the reduced DNA binding in the presence of MG could be caused by a reduction in the amount of the p65 protein.

TNF induces NF-κB activation in many cell types. Therefore, we examined whether the inhibition of the TNF-induced NF-κB activation by methylglyoxal was cell-type specific or if it was a general phenomenon. HUVEC cells as well as HeLa cells

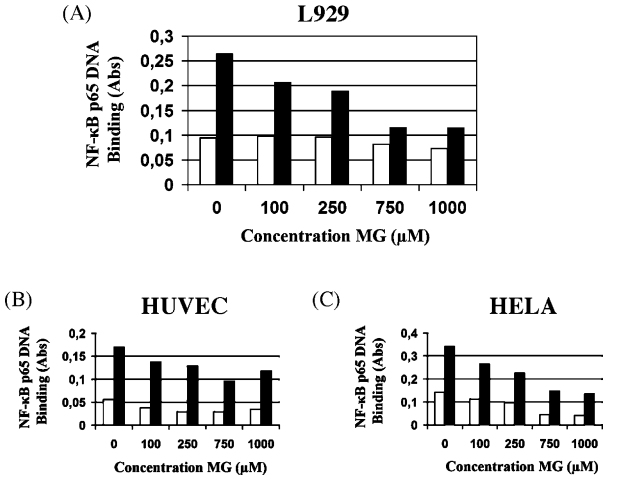

Fig. 1 – Methylglyoxal inhibits TNF-induced NF-κB p65 DNA binding. (A) L929 cells were incubated with TNF (1000 U/ml) for 1 h in the presence of increasing concentrations of MG. Cellular extracts were prepared as described in Section 2. The DNA-binding activity of NF-κB was determined by a colorimetric NF-κB p65 Transcription Factor Assay (Chemicon®). Absorbance was measured at 450 nm. White bars: control treated cells; black bars: TNF-treated cells. (B and C) Inhibition of the TNF-induced NF-κB p65 DNA binding activity by MG in HUVEC (B) and HeLa cells (C).

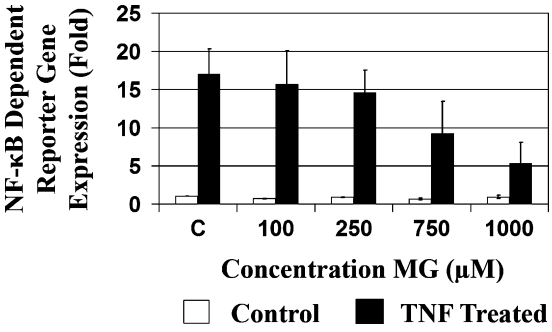

Fig. 2 – Methylglyoxal inhibits TNF-induced NF-κB-dependent reporter gene expression. L929 cells stably transfected with a NF-κB-binding sites-containing reporter plasmid and an internal control plasmid (see Section 2) were treated with TNF (1000 U/ml) for 3 h in the presence of increasing concentrations of methylglyoxal. Lysates were assayed for reporter gene expression and normalized for protein concentration. The average of three independent experiments of the TNF-induced luciferase activity is shown as 'fold induction'.

were treated with TNF (1000 U/ml) for 1h in the presence of increasing concentrations of MG. As shown in Fig. 1B and C, respectively, inhibition of the TNF-induced p65 DNA-binding was already observed at 100 μM MG and maximum inhibition (±45%) was obtained at 750 μM MG. In all three cell lines, no further inhibition of the TNF-induced p65 DNA binding was observed with MG concentration higher than 750 μM, which indicates that the MG-mediated inhibition can be saturated.

3.2. *Methylglyoxal inhibits TNF-induced NF-κB-dependent reporter gene expression*

Because NF-κB DNA-binding does not always correlate with NF-κB-dependent gene transcription [25], we investigated whether methylglyoxal also had an effect on TNF-induced NF-κB-dependent reporter gene expression. For this purpose, we used L929sA cells that were stably transfected with the NF-κB-driven reporter gene construct $(IL6\text{-}κB)_3$-50IL6P-luc + that contains multiple NF-κB responsive elements coupled to a minimal IL-6 promoter in front of the luciferase reporter gene. These cells have been previously described and also contain an internal control plasmid pPGKβGeobpA for the constitutive expression of the (neo)r-β-galactosidase fusion protein [26]. Cells were stimulated with TNF for 3 h in the presence of increasing concentrations of methylglyoxal. Cell lysates were assayed for corresponding reporter gene activity and the expression of the internal control protein. MG (at all concentrations used) had no effect on the expression of the internal control protein (data not shown). The TNF-induced NF-κB-regulated reporter gene expression is shown in Fig. 2. The average of three independent experiments is shown. It is clear that MG also inhibited the TNF-induced NF-κB-dependent reporter gene expression in a concentration-dependent manner. The degree of inhibition was somewhat smaller compared to the MG-mediated inhibition of the NF-κB p65 DNA binding, but this might be due to the longer incubation time with TNF (3 h) in the case of NF-κB-dependent reporter gene expression. Because, longer incubation time may allow for more detoxification of the exogenously added MG through the glyoxalase system, which in turn results in lower intracellular concentrations of MG.

3.3. *Methylglyoxal delays TNF-induced degradation of IκBα and inhibits TNF-induced NF-κB-dependent re-synthesis of IκBα*

TNF-induced NF-κB activation in L929 cells is mediated through the classical pathway, which involves the phosphorylation, the subsequent ubiquitination and proteasomal degradation of its inhibitor IκBα, and the subsequent liberation of the NF-κB dimers (p65/p50 dimers) [27]. Following degradation of IκBα, the p65/p50 heterodimer translocates to the nucleus, where it induces the transcription of its target genes. One of these target genes is its own inhibitor IκBα, and the re-synthesis of IκBα results in an auto-regulatory loop that culminates in the re-inhibition of NF-κB and translocation to the cytoplasm by newly synthesized IκBα [28,29]. MG is highly reactive to Lys, Arg and Cys residues [30]. Thus, a likely mechanism for the MG-mediated inhibition of TNF-induced NF-κB activation could be the inhibition of IκBα degradation via interference with the ubiquitination of Lys residues. Therefore, we investigated whether MG had an effect on the TNF-induced IκBα degradation. Also monitoring the TNF-induced re-synthesis of IκBα allowed us to measure the NF-κB-dependent transcription of one of its endogeneous target genes in the presence of MG. IκBα levels can be easily

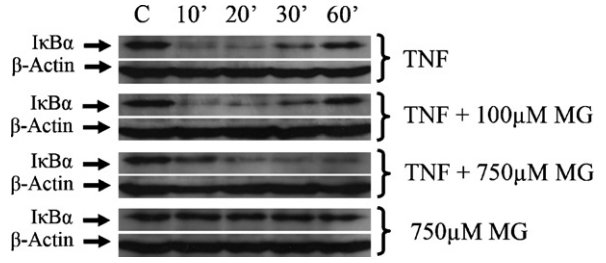

Fig. 3 – Effects of methylglyoxal on the TNF-induced degradation of IκBα and NF-κB-dependent re-synthesis of IκBα. L929 cells were incubated with TNF for the indicated time points in the absence and presence of 100 and 750 μM MG. Total cell extracts were probed for IκBα by Western blotting. β-actin was used as a loading control.

monitored by Western blotting, using a specific anti-IκBα antibody, as shown in Fig. 3. TNF treatment of L929 cells leads to a rapid and drastic degradation of IκBα after only 10 min. Re-synthesis of IκBα is already evident starting at 30 min of TNF treatment (Fig. 3). In the presence of 100 μM MG, the TNF-induced degradation of IκBα was not affected, but the NF-κB-dependent re-synthesis of IκBα was already slightly inhibited. However, in the presence of 750 μM MG, the degradation of IκBα was slowed down, but not inhibited, because at 1 h of TNF treatment IκBα was fully degraded. At this time point, the NF-κB-dependent re-synthesis of IκBα was strongly inhibited in the presence of MG (Fig. 3). Even at 1.5 h of TNF treatment, NF-κB-dependent re-synthesis of IκBα was still inhibited (data not shown), indicating a sustained action of MG. Note, that MG treatment alone had no effect on the IκBα levels. Furthermore, phosphorylation studies of IκBα showed that MG did not affect the TNF-induced phosphorylation of IκBα (data not shown), nor was, the ubiquitination of IκBα considerably affected by MG. This was investigated by immunoprecipitation of IκBα followed by detection with an anti-ubiquitin antibody (data not shown). A plausible explanation for the delayed TNF-induced degradation of IκBα in the presence of higher concentrations of MG could be partial stabilization of IκBα. A similar stabilization of the IκBα protein has also been observed in the presence of nitric oxide [31].

In conclusion, the above data indicate that, at higher concentrations, MG can delay the TNF-induced degradation of IκBα and can strongly inhibit the NF-κB-dependent re-synthesis of its inhibitor IκBα. At lower concentrations (100 μM), MG has no effect on the TNF-induced degradation of IκBα, but the NF-κB-dependent re-synthesis of IκBα is already inhibited. This indicates that the transcriptional activity of NF-κB in the TNF-induced NF-κB activation pathway is the primary target for methylglyoxal.

3.4. *Methylglyoxal delays the TNF-induced nuclear translocation of NF-κB*

Inhibition of NF-κB transcriptional activity could also result from inhibition of its nuclear translocation. This is conceivable as the nuclear localization signal (NLS) in p65 contains several Arg and Lys residues that could be potential target residues for MG-modification [30]. Therefore, we investigated whether MG treatment (750 μM) affected the nuclear translocation of the NF-κB p65. Immuno-cytochemical analysis of the p65 sub-unit was performed by laser confocal immunofluorescence microscopy. As shown in Fig. 4, p65 is primarily located in the cytoplasm in untreated L929 cells as is evident from the presence of large dark voids in the confocal image, which correspond to the nuclei in the transmission image. In TNF-treated cells, p65 is concentrated mainly in the nucleus after only 10 min of TNF treatment (this translocation lasted for at least 30 min). After 1 h of TNF treatment, p65 was already more concentrated in the cytoplasm and the nuclei had already started to become void of p65. This cytoplasmic re-translocation of p65 is due to the NF-κB-dependent re-synthesis of its inhibitor IκBα. However, in the presence of MG the TNF-induced translocation of p65 was delayed, but not inhibited. The p65 remained largely cytoplasmic in the first 20 min of TNF treatment. After only 30 min of TNF treatment, p65 began to concentrate in the nucleus, and, after 1 h of TNF treatment, p65 was fully concentrated in the nucleus. This indicates that the cytoplasmic re-translocation of p65 is also inhibited by the presence of MG. This is most likely due to the lack of NF-κB-dependent re-synthesis of IκBα, which is responsible for the cytoplasmic re-translocation of NF-κB [32]. Significantly, MG's strong inhibition of the DNA-binding activity of the p65 sub-unit was determined at 1 h of TNF treatment—that is, when the p65 sub-unit was fully concentrated in the nucleus in the presence of MG. Taken together, these results indicate that the MG-mediated inhibition of the TNF-induced NF-κB DNA-binding is not due to inhibition of its nuclear translocation. Furthermore, these results suggest that the inhibitory effect of MG on the TNF-induced NF-κB DNA binding and the NF-κB-dependent transcription is rather executed at the level of the transcription factor itself. This is not so unlikely, as it has been reported that certain NF-κB inhibitors, such as for example NO, suppress NF-κB activation by directly blocking the binding of NF-κB to the DNA [33,34].

3.5. *Methylglyoxal does not affect the TNF-induced phosphorylation of p65*

Optimal NF-κB activation is regulated by phosphorylation of the NF-κB proteins themselves (reviewed in [35]). TNF induces phosphorylation of p65, which is required for its transcriptional activity, but not for DNA-binding [35]. Phosphorylation on Ser536, which is located in the transactivation domain, is mediated by IKKβ. Because p65 was fully clustered in the nucleus upon TNF stimulation in the presence of MG, we also wanted to investigate whether MG had an effect on the TNF-induced transactivation of p65. The TNF-induced phosphorylation of p65 in L929 cells in the presence and absence of methylglyoxal was analysed by Western blotting using phopho-specific antibodies against p65. As shown in Fig. 5, TNF induces a clear phosphorylation on Ser 536. However, in the presence of 750 μM MG, this TNF-induced phosphorylation of p65 is not affected, indicating that MG did not interfere with the TNF-induced transactivation of p65. Along the same line, TNF-induced phosphorylation on Ser 276 [36], which is located in the Rel homology domain of p65, was not affected in the presence of 750 μM MG. These data further suggested that the inhibitory effect of MG on the p65 DNA

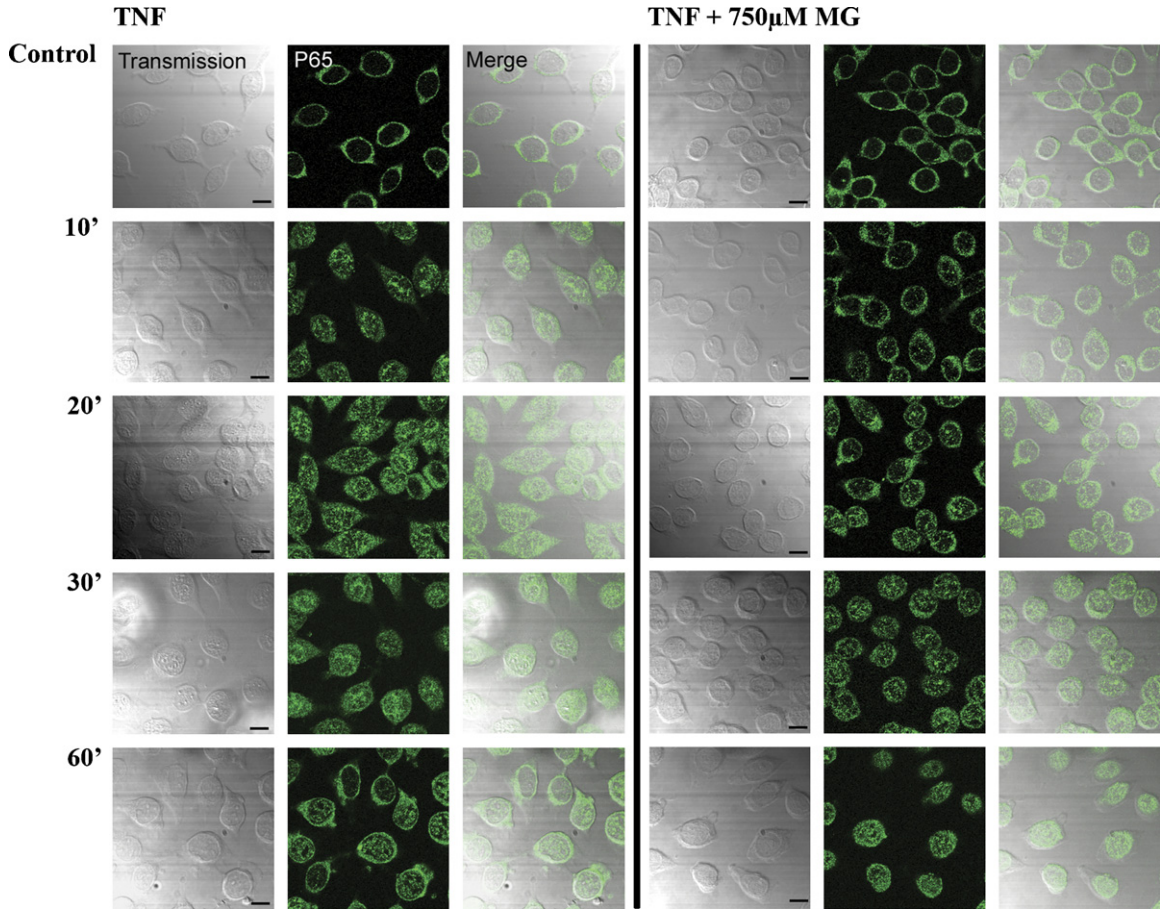

Fig. 4 – Methylglyoxal delays, but does not inhibit, the TNF-induced nuclear translocation of NF-κB p65. L929 cells were incubated with TNF for the indicated time points in the absence and presence of 750 μM MG. Samples were prepared for immunocytochemistry using anti-p65 antibody and Alexa-488-conjugated anti-mouse IgG. Representative confocal images are shown. The transmission images are shown in the left panels, the immunofluorescence of the NF-κB p65 sub-unit is shown in the middle panels, and the overlay is shown in the right panels. The scale bar is 10 μm.

binding and NF-κB-dependent reporter gene expression must be executed at the level of p65 itself.

3.6. *Involvement of Cys 38 in NF-κB p65 for inactivation by methylglyoxal*

Members of the NF-κB family, including p65, are redox-regulated transcription factors. This redox regulation is mediated by the redox state of a cysteine residue located in the N-terminal conserved region that is responsible for DNA binding in all NF-κB family members and corresponds to C38 in p65 [37–39]. NF-κB must be in a reduced state to bind DNA *in vitro* [40]. The C38 residue is located within a polypeptide loop used to make many of the specific contacts with the κB motif DNA and contacts a phosphate in the DNA backbone [41,42]. Furthermore, inhibition of the NF-κB p50 DNA binding activity by nitric oxide (NO) is mediated through NO-modification of this conserved Cys 62 residue, and the DNA binding of a p50 C62S mutant is much more resistant to inhibition by NO (28). MG can react with Cys residues to form a hemithioacetal [30] and furthermore, MG has been shown to be involved in the regulation of the yeast transcription factor Yap1 via modification of Cys residues [43]. This prompted us to investigate whether the conserved residue Cys 38 in p65 was involved in the methylglyoxal-mediated inhibition of the NF-κB DNA binding. To this end, we made use of the fact that transient transfection with NF-κB p65 by itself is sufficient to induce NF-κB DNA binding activity [44], and TNF stimulation of these transfected cells did not further increase the p65 DNA binding activity (data not shown). NF-κB p65 WT and a C38A mutant were transiently overexpressed in HeLa cells. Twenty-four

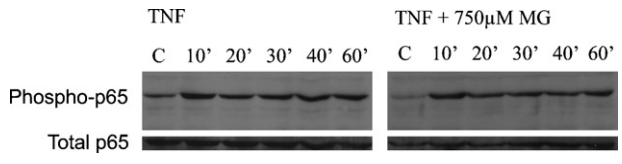

Fig. 5 – Methylglyoxal does not affect the TNF-induced phosphorylation of p65. L929 cells were incubated with TNF (1000 U/ml) for the indicated time points in the absence and presence of 750 μM MG. Total cell extracts were analysed by Western blotting using an antibody that specifically recognizes p65 phosphorylated on Ser 536. The same blot was than re-probed with an anti-p65 antibody.

hours after transfection, the cells were treated with increasing concentrations of MG (as indicated in Fig. 6) for 1 h. Total cell lysates were prepared in Totex buffer, and the DNA binding activity was determined with a quantitative transcription factor assay as described above. The expression levels of the ectopically expressed p65 WT and mutant protein were analysed by Western blotting and were found to be comparable and not affected by MG treatment (Fig. 6A). Mock-transfected cells were used to determine the DNA binding activity of endogeneous p65, which was negligible compared to the activity of the ectopically expressed proteins (Fig. 6B). Three independent experiments were performed and a representative experiment is shown in Fig. 6B. The p65 C38A mutant protein had already reduced DNA-binding compared to the WT p65, but DNA binding was still sufficient to measure the effect of MG. MG treatment of HeLa cells transfected with p65 WT resulted in a drastic inhibition of its DNA binding activity, with at least 50%–60% inhibition at 100 μM MG and 90% at 750 μM MG. However, the p65 C38A mutant was considerably more resistant to inhibition of its DNA binding activity by MG as compared to the WT protein (Fig. 6B). The relative inhibition (mean of three independent experiments) of DNA binding activity of the WT and mutant protein is shown in Fig. 6C. The DNA binding of the p65 C38A mutant was only 20% to maximum 25% inhibited by 100 μM MG. The mutant protein was thus 50%–60% more resistant to inhibition by MG (100 μM) as compared to the WT protein (Fig. 6C). Also, at higher concentrations of MG, the mutant protein was still 50% more resistant to inhibition by MG.

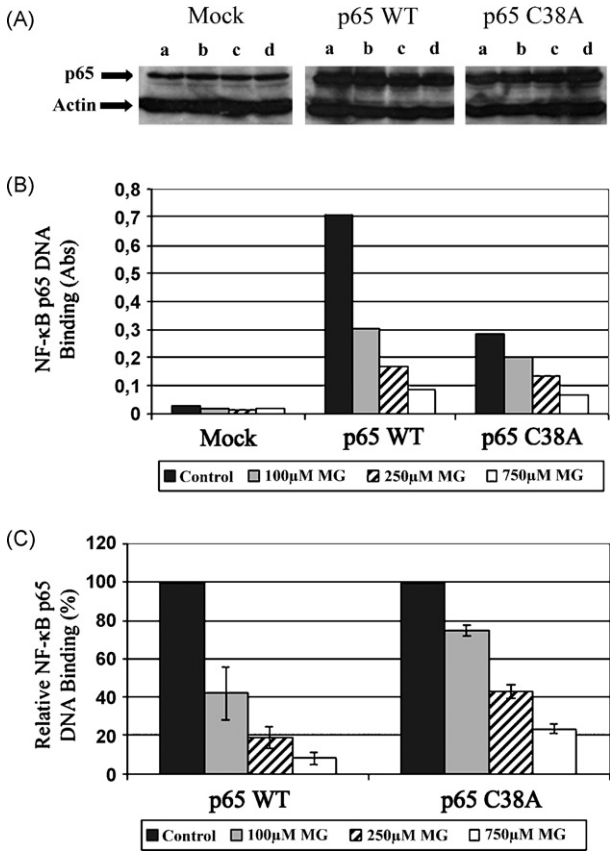

Fig. 6 – Reduced sensitivity of the p65 C38A mutant towards inhibition of DNA binding activity by methylglyoxal. (A) Western blot with anti-p65 antibody. Transient expression level of p65 WT and p65 C38A mutant in HeLa cells. Expression levels of endogeneous p65 are also shown. a: untreated, b: 100 μM MG, c: 250 μM MG, d: 750 μM MG. Note that the expression level of the p65 proteins was not affected by MG treatment. An anti-actin antibody was used as control for equal protein load. (B) Inhibition of DNA binding activity by MG of the p65 WT and the p65 C38A mutant. The absorbance values at 450 nm are shown of one representative experiment. The DNA binding activity of endogeneous p65 in mock-transfected cells is negligible compared to that of the ectopically expressed proteins. (C) Relative inhibition of DNA binding activity by MG of the p65 WT and the p65 C38A mutant. The mean of three independent experiments is shown.

In conclusion, these data indicate that Cys 38 in NF-κB p65 is involved in the MG-mediated inhibition of the DNA binding activity. Furthermore, these data suggest that MG may directly modify Cys 38 in NF-κB p65 and that MG-modification of the transcription factor is a very site-specific and targeted process.

3.7. *Overexpression of NF-κB p65 increases the synergistic effect of methylglyoxal on TNF-induced cell death*

Since NF-κB activation inhibits TNF-induced cell death, we wanted to further explore the role of NF-κB activation in the synergistic action of methylglyoxal on TNF-induced cell death. For this, we used overexpression of NF-κB p65, which results automatically in NF-κB activation. Because there is a large clonal variability in sensitivity to TNF-induced cell death in L929 cells, we used an inducible expression system so that TNF sensitivity can be compared in the same clone upon induced expression of p65, circumventing the problem of clonal variability. Murine fibrosarcoma L929 cells were transfected with pSP64Mx NF-κB p65. In this expression vector, p65 is under the control of the murine Mx promotor, which is inducible with interferon α (IFNα) [45]. After transfection, G418-resistant clones were screened and retained when they showed low-level leak expression in the non-induced condition and strong expression in the induced condition. Four clones for p65 and four mock clones were selected for use in further experiments. For induction, cells were incubated with 500 IU/ml IFNα for 16 h prior to TNF treatment. Levels of ectopically expressed p65 protein in the non-induced and induced conditions and the endogenous levels of p65 in the mock clones are shown in Fig. 7A. Compared to the mock clones, the p65-expressing clones had a leaky expression in the non-induced condition, a phenomenon that we had previously observed with this inducible expression system in L929 cells [23]. As expected, overexpression of p65 resulted in its increased DNA binding activity in untreated cells as measured by the non-radioactive NF-κB p65 Transcription Factor Assay (data not shown). TNF-treatment of these clones did not strongly activate NF-κB, because basal NF-κB activity was already very high in these clones.

TNF-induced cell death in the clones was measured as a function of time by flow cytometry using the uptake of

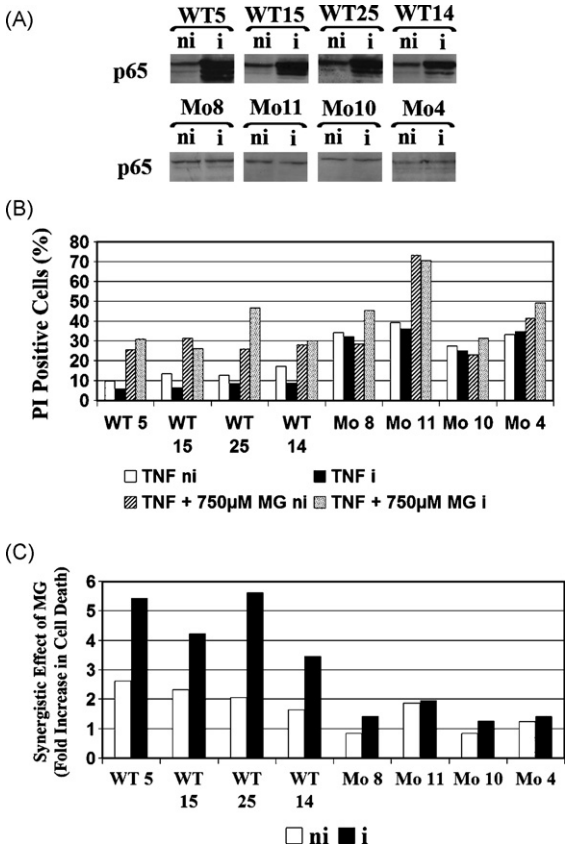

Fig. 7 – Overexpression of NF-κB p65 sensitizes for the synergistic action of methylglyoxal on TNF-induced cell death. (A) Expression levels of ectopically expressed p65 (WT clones) and endogenously expressed p65 (Mock clones) in the induced (i) and the non-induced (ni) condition. Note that all p65 WT clones (WT) have leak expression in the non-induced condition, as compared to the expression level of endogenous p65 in the mock clones (Mo). Equal amounts of protein were loaded for all clones. (B) Percentage of TNF-induced cell death expressed as the percentage of PI-positive cells after 4 h of TNF treatment (100 U/ml) of the different clones in the non-induced (ni) and the induced (i) condition and in the absence and presence of MG. (C) Synergistic effect of methylglyoxal on TNF-induced cell death of the different clones in the non-induced and the induced condition for the same experiment as shown in (B). The synergistic effect is expressed as the fold-increase in percent of TNF-induced cell death in the presence of MG over the percent of TNF-induced cell death without MG for the same condition.

propidium iodide as a measure for cell death [46]. The experiments were repeated three times and produced similar results each time. Fig. 7B depicts a representative experiment showing the percentage of cell death for the various clones after 4 h of TNF treatment (100 U/ml) under the non-induced and induced condition and in the presence and absence of MG. The percentage of cell death in all control conditions was negligible and is therefore not shown. As it is clear from Fig. 7B, all p65 clones – in the induced as well as in the non-induced condition – are considerably more resistant to TNF-induced cell death compared to the mock clones. The low sensitivity for TNF-induced cell death of these clones in the non-induced condition is most probably due to constitutive NF-κB activity caused by leaky expression of p65. Induced overexpression of p65 in these clones caused a further decrease (average of 40%) in TNF-induced cell death. These data agree with the long-known fact that NF-κB activation leads to inhibition of TNF-induced cell death [20,47,48]. As we have previously shown [23], IFNα treatment of mock clones had no significant effect on TNF-induced cell death, which excluded the possibility that inhibition of TNF-induced cell death in the p65 clones was solely due to IFNα treatment.

The synergistic effect of MG (750 μM) on TNF-induced cell death in the various clones in the induced and non-induced conditions is presented in Fig. 7C as the fold increase in % of TNF-induced cell death in the presence of MG over the % of TNF-induced cell death without MG for the same condition. The degree of synergism of MG on TNF-induced cell death in the mock clones is comparable to that of parental L929 cells, which we have described previously [12]. However, upon overexpression of NF-κB p65, the cells became extremely sensitized for TNF-induced cell death in the presence of MG. The average fold increase in TNF-induced cell death was 4.6 in the p65 clones in the induced condition, compared to an average of 1.5 in the mock clones. Furthermore, the fold increase in TNF-induced cell death caused by MG was not considerably different in the induced and non-induced conditions in the mock clones, while the synergistic effect of MG on TNF-induced cell death in the p65 clones was even more pronounced upon induced overexpression of p65.

L929 clones that overexpressed the p65 C38A mutant protein were also generated. These clones could still be sensitized by MG for TNF-induced cell death to an extent similar to that of the L929 clones overexpressing the p65 WT protein (data not shown). Similar to the WT p65 clones, untreated cells expressing the p65 C38A protein also had a high basal NF-κB activity as measured by DNA-binding, because mutant p65 still binds DNA, but to a lesser extent than the WT protein (see Fig. 6B). High basal NF-κB activity (either in p65 WT clones or p65 C38A clones) leads to induction of its target genes – a number of anti-apoptotic genes (among others) – before the cells are even treated with TNF. This makes them more resistant to TNF-induced cell death, but at the same time these cells are much more sensitized by MG for TNF-induced cell death. These results indicate that the sensitizing effect of MG on TNF-induced cell death in cells with constitutive NF-κB activity cannot be explained solely by inhibition of the p65 DNA-binding activity, but that MG may also act on NF-κB target proteins that play a role in conferring resistance to TNF-induced cell death. Yet, the nature of these target proteins remains to be determined.

In summary, these data indicate that more NF-κB activation leads to stronger synergistic action of MG on TNF-induced cell death. This is particularly interesting for anti-tumor therapy, as many tumors have constitutive NF-κB activity that confers resistance to chemotherapy- or radiotherapy-induced cell death [27]. Therefore, agents that could increase the intracellular concentration of MG, such as inhibitors of the

non-phosphorylated form of GLO1, may increase the therapeutic effectiveness when used in combination with TNF-, chemo- or radio-therapies.

4. Discussion

In the current study, we show that methylglyoxal inhibits TNF-induced NF-κB DNA binding and NF-κB-dependent reporter gene expression in a concentration-dependent manner. Furthermore, we provide evidence that this MG-mediated inhibition of TNF-induced NF-κB DNA binding is not due to inhibition of the degradation of its inhibitor IκBα. At low concentrations, MG had no effect on the TNF-induced degradation of IκBα, but the NF-κB-dependent re-synthesis of IκBα was already inhibited. However, at higher concentrations, MG caused a delayed degradation of IκBα, but the degradation was not inhibited because no residual amount of IκBα remained after 1 h of TNF treatment. At this time point, the TNF-induced NF-κB-dependent re-synthesis of IκBα was strongly inhibited in the presence of higher concentrations of MG. A conceivable explanation would be that MG had an inhibitory effect on IKKβ, the catalytic subunit of the kinase that is responsible for the phosphorylation of IκBα, because the TNF-induced activation of IKKβ can be inhibited by NO through S-nitrosylation of Cys residue 179 of IKKβ [49]. Therefore, it would be plausible that MG could also modify this residue and interfere with the phosphorylation of IκBα. However, this explanation is not in line with our data, because the phosphorylation of IκBα was not affected by the higher concentration of MG, nor was the TNF-induced phosphorylation of p65 on Ser 536 affected, which is also mediated by IKKβ [35]. A possible explanation for the delayed degradation of IκBα could be stabilization of the IκBα protein, an effect that has also been observed in the presence of nitric oxide [31]. The delayed degradation of IκBα by MG was also reflected by a delayed translocation of NF-κB to the nucleus. Significantly, the DNA-binding activity of the NF-κB p65 sub-unit was strongly inhibited in the presence of MG, even when the NF-κB p65 sub-unit was fully clustered in the nucleus upon TNF-stimulation. Furthermore, we show that the conserved Cys 38 residue that is located in the DNA binding loop of p65 is involved in the MG-mediated inhibition of the DNA binding activity of p65. The p65 C38A mutant protein was 50% more resistant, compared to the WT protein, to inhibition by MG. The fact that the C38A mutant could still be partially inhibited by MG might be due to MG-modification of Arg or Lys residues, which are located in the DNA-binding loop and involved in the specific contacts with DNA [50]. The DNA binding region containing the Cys 38 residue is conserved in all NF-κB family members and makes many of the specific contacts with the κB motif DNA. The Cys 38 residue contacts a phosphate in the DNA backbone and is responsible for the redox regulation of NF-κB [41,42]. This conserved Cys residue needs to be in the reduced state to bind DNA [40]. For instance, it has been shown that NO can also inhibit the DNA binding activity of NF-κB p50 by NO-modification of the Cys 62 residue [51]. Furthermore, thioredoxin regulates the DNA-binding activity of NF-κB by reduction of a disulphide bond involving this conserved Cys residue [52]. Because methylglyoxal can react with Cys residues in proteins to form a hemithioacetal, it is quite conceivable that MG directly modifies the Cys 38 residue in NF-κB p65, thereby inhibiting the contact with DNA. Our results are also in line with the recently described inhibitory effects of Plumbagin on the TNF-induced NF-κB activation. Plumbagin is a potential anti-cancer agent (derived from the medicinal plant *Plumbago zeylanica*) that inhibits the TNF-induced NF-κB p65 binding to DNA. This inhibition is also mediated through the conserved Cys 38 residue in the DNA binding loop of p65 [53].

Our data are also in line with recent data obtained from yeast, where it has been shown that MG directly modifies Cys residues in the oxidative-stress responsive transcription factors Yap1 and Pap1 in *S. cerevisiae* and *S. pombe*, respectively [43,54]. Yap1 is the functional homologue of mammalian AP-1, whose DNA-binding activity also depends on the redox regulation of a conserved Cys residue [55]. Direct MG-modification of Yap1 is sufficient for translocation to the nucleus and activation of its target genes, thus indicating that MG regulates the transcription factor positively [43]. Furthermore, in retinal Müller cells, increased concentrations of methylglyoxal, caused by hyperglycemia, have been linked to increased expression of angiopoietin-2. This is caused by MG-modification of the co-repressor mSin3A [56]. All these data point to a general role for methylglyoxal in the regulation of transcription factors.

It is worth noting that the promotor region of human glyoxalase 1 contains consensus sites for NF-κB, as well as for AP-1 [57], suggesting that MG may play a role in the redox regulation of these transcription factors in both physiological and pathophysiological processes. Furthermore, the concentrations of MG needed to inhibit the DNA binding of NF-κB p65 could be in the physiological range, as it has been reported that intracellular concentrations of MG in normal growing cells can be up to 300 μM [58]. In addition, in plasma of diabetic patients, MG concentrations of up to 400 μM have been reported [59]. In L929 cells, partial (20%) inhibition of p65 DNA binding was observed at 100 μM MG. Maximum inhibition (±80%) in these cells is observed at 750 μM, while in HUVEC and HeLa cells no more than ±45% inhibition could be obtained at high concentrations of MG. Thus, the sensitivity of NF-κB p65 to MG seems to be strongly cell-type dependent and may be dependent on the expression levels of glyoxalase 1, the enzyme responsible for detoxification of MG. For the moment, it remains to be determined whether MG-modified p65 occurs *in vivo*. Currently, the detection of this is hampered by a lack of the proper tools such as antibodies that specifically recognize MG-modified p65 on residue Cys 38.

More and more, NF-κB is being considered an important target for cancer therapy, as its activation is one of the major obstacles on the road to tumor cell death (reviewed in [60,61]). For instance, it has been shown that tumors with constitutive NF-κB activity usually show increased resistance to radiation and chemotherapy [62]. Inhibition of NF-κB not only leads to enhanced apoptosis but also to increased sensitivity to radiation and chemotherapy [60]. Furthermore, in this paper we show that increased NF-κB activation, induced by over-expressing p65, leads to stronger synergistic action of MG on TNF-induced cell death. Our data suggest that the sensitizing effect of MG on TNF-induced cell death in cells with

constitutive NF-κB activity cannot be explained solely by inhibition of the p65 DNA-binding activity, but that MG may also act on NF-κB target proteins that play a role in conferring resistance to TNF-induced cell death. In addition, our data may explain why overexpression of GLO1 is involved in resistance to chemotherapy-induced tumor cell death [11]. Overexpression of GLO1 leads to excessive detoxification of MG, and thus to concentrations that are too low to modify NF-κB and inhibit its DNA binding activity. Therefore, agents that could increase the intracellular concentration of MG, such as inhibitors of the of GLO1, may increase the therapeutic effectiveness when used in combination with TNF-, chemo- or radio-therapies.

In conclusion, the data presented in this report indicate that the glycolytic metabolite methylglyoxal provides another control mechanism for modulating the expression of NF-κB-responsive genes. Furthermore, MG may be responsible for tipping the balance towards TNF-induced cell death by suppressing NF-κB activation and/or by acting on NF-κB target proteins. This may be one of the molecular mechanisms responsible for the deteriorating effect of methylglyoxal and its role in the pathophysiology of several diseases.

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