Up-regulation of base excision repair correlates with enhanced protection against a DNA damaging agent in mouse cell lines


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ABSTRACT

DNA polymerase \( \beta \) is required in mammalian cells for the predominant pathway of base excision repair involving single nucleotide gap filling DNA synthesis. Here we examine the relationship between oxidative stress, cellular levels of DNA polymerase \( \beta \) and base excision repair capacity in vitro, using mouse monocytes and either wild-type mouse fibroblasts or those deleted of the DNA polymerase \( \beta \) gene. Treatment with an oxidative stress-inducing agent such as hydrogen peroxide, 3-morpholinosydnonimine, xanthine/xanthine oxidase or lipopolysaccharide was found to increase the level of DNA polymerase \( \beta \) in both monocytes and fibroblasts. Base excision repair capacity in vitro, as measured in crude cell extracts, was also increased by lipopolysaccharide treatment in both cell types. In monocytes lipopolysaccharide-mediated up-regulation of the base excision repair system correlated with increased resistance to the monofunctional DNA alkylating agent methyl methanesulfonate. By making use of a quantitative PCR assay to detect lesions in genomic DNA we show that lipopolysaccharide treatment of fibroblast cells reduces the incidence of spontaneous DNA lesions. This effect may be due to the enhanced DNA polymerase \( \beta \)-dependent base excision repair capacity of the cells, because a similar decrease in DNA lesions was not observed in cells deficient in base excision repair by virtue of DNA polymerase \( \beta \) gene deletion. Similarly, fibroblasts treated with lipopolysaccharide were more resistant to methyl methanesulfonate than untreated cells. This effect was not observed in cells deleted of the DNA polymerase \( \beta \) gene. These results suggest that the DNA polymerase \( \beta \)-dependent base excision repair pathway can be up-regulated by oxidative stress-inducing agents in mouse cell lines.

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical, hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and nitric oxide (NO), arise during normal cellular metabolism and especially in association with inflammatory events (1–3). Some mammalian tissues frequently encounter oxidative DNA damage by ROS and disorders such as cancer and atherosclerosis have been suggested to be associated with ROS-mediated DNA damage (4,5). Oxidative lesions in DNA are heterogeneous and include strand breaks, abasic sites (AP sites) and oxidized bases and sugars (6,7). Repair of such DNA lesions may occur by various DNA repair pathways, but is mainly by the base excision repair (BER) pathway in the cases of AP sites and oxidized bases (6,7). Here we evaluate the hypothesis that cellular resistance to a genotoxic stress can be adaptively up-regulated in mammalian cells and assess the BER capacity of mouse cells as a function of treatment with oxidative stress-inducing agents. Five enzyme activities are involved in the DNA polymerase \( \beta \) (\( \beta \)-pol)-dependent ‘short patch’ or single nucleotide BER pathway: DNA glycosylase, AP endonuclease, dRpase, DNA polymerase and DNA ligase. In mammalian cells \( \beta \)-pol appears to contribute both the dRpase and gap filling DNA synthesis activities, after action by DNA glycosylase and AP endonuclease (6–10).

Cells can be activated to express ROS by endogenous cytokines and by oxidative stress-inducing agents, such as lipopolysaccharide (LPS), which induces production of NO (11–22). We report here that LPS treatment causes an increase in the cellular \( \beta \)-pol level and in the in vitro BER capacity of monocyte and wild-type fibroblast cell lines. This LPS-mediated up-regulation of BER in monocytes correlates with increased resistance to the alkylating agent methyl methanesulfonate (MMS). In addition, LPS treatment is also associated with a decrease in endogenous genomic DNA damage, again possibly through enhanced DNA repair. In studies of \( \beta \)-pol null/BER-deficient fibroblast cells, LPS treatment was associated with accumulation of DNA damage. These results illustrate an example in mammalian cells of altered DNA repair

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gene expression rendering cells less susceptible to endogenous plus exogenous genotoxic stress.

MATERIALS AND METHODS

Reagents

LPS (Escherichia coli 0111:B4), H2O2, 3-morpholinopropionic acid, (SIN-1), xanthine/xanthine oxidase (X/XO), creatine phosphokinase, diTris–phosphocreatine, MMS and nicotinamide adenine dinucleotide (NAD) were obtained from Sigma Chemical Co. (St Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), gentamicin, heat-inactivated fetal bovine serum, λHindIII DNA and penicillin/streptomycin were from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). Fetal bovine serum was obtained from Hyclone Laboratories Inc. (Logan, UT). dNTPs were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Hygromycin B and non-acetylated bovine serum albumin (BSA) were from Boehringer Mannheim (Indianapolis, IN); [α-32P]dNTPs were from DuPont NEN (Boston, MA). Oligonucleotides were from Operon Technologies (Alameda, CA). The mouse monocyte cell line (PU5-1.8) was obtained from American Type Culture Collection (Rockville, MD). Wild-type (Mβ16tsA, clone 1B5) and β-pol-deleted (Mβ19tsA, clone 2B2) mouse embryonic fibroblasts were provided by this laboratory (10).

Cell culture

Mouse PU5-1.8 monocyte cells were grown in a humidified atmosphere with 10% CO2 in DMEM containing gentamicin (50 µg/ml), L-glutamine (4 mM) and heat-inactivated fetal bovine serum (10%) at 37°C. For experiments cells were split by manual disruption, centrifuged and resuspended in DMEM with 0.5% fetal bovine serum and, unless otherwise stated, plated in 100 mm dishes at a concentration of 2 × 106 cells/dish and grown for 20–24 h. At initiation of the experiment cells were replenished with fresh medium containing 0.5% fetal bovine serum with or without oxidative stress-inducing agents. For the mouse fibroblast cell lines, cells were grown and treated with oxidative stress-inducing agents in medium with 10% serum under culture conditions as described previously (10).

Western blotting

After stimulation of the cells with oxidative stress-inducing agents as described in the text and figure legends cell-free extracts were prepared at the indicated time points as described (23) with minor modifications. Briefly, cells were scraped and centrifuged at 500 g for 5 min at 4°C and then washed once with ice-cold phosphate-buffered saline (PBS). The cells were lysed in a buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2.7 µg/ml aprotinin. The lysates were microcentrifuged at 11 000 r.p.m. for 10 min to remove any cellular debris. The cell-free supernatant fractions were stored at −70°C for later analysis of β-pol. Total cellular protein (20 µg) was separated by electrophoresis in a 12.5% SDS–polyacrylamide gel and electro-transferred to nitrocellulose membrane. β-Pol was measured by incubating the membrane with the mouse anti-β-pol monoclonal antibody 18S followed by an antibody to mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (23). Immobilized HRP activity was detected by enhanced chemiluminescence (Amersham). The absolute amount of β-pol protein in cell extracts was calculated on the basis of a standard curve derived from plotting pure recombinant rat β-pol concentration versus integrated optical density as described previously (23).

Base excision repair

For measuring BER activity cell-free extracts were prepared by resuspending cells in PBS containing 1 mM PMSF, 1 mM diethiothreitol, 2.7 µg/ml aprotinin and lysing cells by four cycles of freeze/thawing. The cell lysate was centrifuged as described above and 5 µg supernatant fraction were used for determination of BER activity according to the method of Singhal et al. (8). Briefly, the standard reaction mixture (20 µl) contained 100 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 1 mM diethiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM NAD, 5 mM diTris–phosphocreatine, 10 U creatine phosphokinase, 40 mM duplex oligonucleotide and 0.3 µM [α-32P]dCTP (sp. act. 6.6 × 106 d.p.m./pmol). Reaction mixtures were incubated for 10 min at 37°C and reactions were stopped by addition of ‘stopping solution’ (0.01% xylene cyanol, 0.01% bromophenol blue and 80% formamide). After incubation at 75°C for 5 min DNA was resolved by electrophoresis in a 15% polyacrylamide gel containing 7 M urea, 89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.0. Gels were dried and autoradiographed at −70°C.

Cell sensitivity assays

Mouse PU5-1.8 monocytes were seeded in 6-well dishes (105 cells/well) in 1.5 ml DMEM containing 0.5 or 10% heat-inactivated fetal bovine serum. After 20–24 h 0.5 ml medium was added to each well, resulting in exposure of the cells to 5 µg/ml LPS in medium with 0.5 or 10% heat-inactivated fetal bovine serum. After 4 h control and LPS-exposed cells were dosed with dilutions of MMS, added to the wells in 1 ml medium containing heat-inactivated fetal bovine serum such that the final medium concentration was 0.5% MMS. The cells were cultured (40 000 cells/35 mm dish) for 4 days continuously in the presence of MMS. Then the cells were counted either by a whole cell or cell lysis procedure (24). Preliminary growth curves had demonstrated that untreated monocytes grow exponentially under these conditions. Results are expressed, i.e. percent control growth, as the number of MMS-treated cells relative to the number of control cells.

Mouse fibroblasts (wild-type, Mb16tsA, clone 1B5; β-pol null, Mb19tsA, clone 2B2) were cultured (40 000 cells/35 mm dish) for 24 h and then treated with LPS (5 mg/ml) in fresh medium containing 10% heat-inactivated fetal bovine serum. After 4 h the medium was removed and fresh medium without LPS was added. Cells were then cultured for an additional period of 4 or 14 h, as indicated in Figure 5, prior to MMS treatment. For this the medium was removed and fresh medium containing MMS was added (2 mM for Mb16tsA and 1 mM for Mb19tsA). After 1 h the cells were washed and fresh medium without MMS was added. Cells were then grown for 3 days and the cell number determined by Coulter counter. The results are expressed as cell number per dish.

DNA isolation and quantitative PCR

High molecular weight DNA from mouse monocyte and embryonic fibroblast cells (5 × 105) was isolated with the
The concentration of total cellular DNA was determined by ethidium bromide fluorescence with an A4-Filter Fluorimeter (excitation bandpass filter at 365 nm and emission cut-off filter at 600 nm) using λHindIII DNA as a standard.

Quantitative amplification of β-pol and β-pol target sequences was performed in either a GeneAmp® PCR System 9600 or 2400 respectively, using the GeneAmp® XL PCR Kit (Perkin-Elmer). Reaction mixtures contained 15 ng template DNA, 1.1 mM Mg(OAc)2, 100 µg/ml non-actetylated BSA, 0.2 mM deoxy-nucleotide triphosphates, 0.2 µM primers, 0.2 µM [α-32P]dATP (sp. act. 6.6 × 10⁶ d.p.m./pmol) and 1 U Tth DNA polymerase. The primers used in this study resulted in PCR amplification of either a 6.5 kb β-pol product or an 8.7 kb β-pol product. The primer sequences for the β-pol gene were MBFOR1 (5′-TATCTCTCTCTCCACTCTCCACCTCCCGT-3′) and MBEX1B (5′-CTGATTGCCGGTGTGACCTCCT-3′) and for the β-pol gene N357 (5′-TTGACGTGATTGGCAATGCCT-3′) and N354 (5′-CTTTATGCCTGCCACCCGGACACT-3′; GenBank accession no. X14061).

PCR was initiated with a 75 °C hot start addition of the polymerase and an initial denaturation at 94 °C for 1 min, followed by 25 cycles of 94 °C denaturation for 15 s and primer extension at 68 °C for 12 min. A final extension at 72 °C was performed for 10 min at completion of the profile. To ensure quantitative conditions a control reaction containing 7.5 ng template DNA was included with every amplification. An aliquot of each reaction mixture after PCR, in 6× loading buffer, was resolved by electrophoresis in TBE buffer at 80 V (5 V/cm) for 4 h in a 1% vertical agarose gel. Afterwards the gels were washed with distilled water, vacuum dried, exposed to a phosphorimager screen for 12–18 h and signals quantified with ImageQuant™.

### Calculation of DNA lesion frequency

DNA lesion frequencies were calculated by dividing the amount of amplification from damaged samples (A₀) by the amount of amplification from non-damaged controls (A₀). A rounded random distribution of lesions and using the Poisson equation 

\[ f(x) = e^{-λ} \frac{λ^x}{x!}, \]

where \( λ \) is the average lesion frequency for the non-damaged control (i.e. the zero class, \( x = 0 \)), the lesion frequency per DNA fragment was determined:

\[ λ = -\ln \frac{A₀}{A₀}. \]

### RESULTS

#### Up-regulation of β-pol by oxidative stress-inducing agents

To examine whether oxidative stress-inducing agents can up-regulate β-pol expression in mouse monocyte and fibroblast cell lines we treated cells with several of these agents and the results are summarized in Table 1. An increase in β-pol expression was observed in both cell lines after exposure to all the agents (Table 1), but among those tested LPS treatment resulted in the greatest increase in cellular level of β-pol. In an experiment to determine the time course of induction by LPS the highest β-pol level was found at 4 h for monocytes and at 8 h for fibroblasts (Fig. 1A and C). To examine the LPS concentration requirement for this β-pol increase, cells were treated with different concentrations of LPS (Fig. 1B and D). In both cell lines 5 µg/ml LPS resulted in maximal up-regulation of β-pol.

#### Effect of LPS on base excision repair activity in vitro

Previous studies have indicated that β-pol is responsible in vitro for uracil-initiated ‘single nucleotide’ gap filling DNA synthesis in bovine tissue and mouse cell extracts (8,10). Since LPS treatment up-regulates expression of β-pol in monocytes and fibroblasts, we examined the effect of LPS treatment on the short patch BER capacity of cell extracts. A 51 bp oligodeoxyribonucleotide containing a G:U mismatch at position 22 was used as substrate (8).

In this assay removal of the uracil residue and replacement with a phosphate group was observed earlier between phorbol 12-myristate 13-acetate and N-methyl-N′-nitro-N-nitrosoguanidine in CHO cells (23).

#### Effect of LPS on monocyte sensitivity to MMS

It has been shown previously that mouse fibroblasts deficient in BER capacity (by virtue of β-pol gene deletion) are more sensitive than wild-type cells to MMS, as well as several other monofunctional DNA alkylating agents (10). Based upon this correlation, we tested LPS-treated monocytes to see if the increase in BER capacity would correlate with an increased...
resistance of cells to MMS. As a control in these experiments we used monocytes growing in medium containing 10% heat-inactivated fetal bovine serum, where LPS treatment does not produce a change in either β-pol level or BER capacity in vitro. In the presence of 10% serum MMS sensitivity was identical in LPS-treated and untreated cells (Fig. 3A). In contrast, when cells were grown under conditions where BER is up-regulated by LPS treatment (0.5% serum) there was an increase in resistance to MMS (Fig. 3B). Finally, it is noted that the difference in serum conditions per se was not associated with a difference in β-pol level; the level of β-pol in untreated cells was similar for cells grown in 10 or 0.5% serum. In summary, these experiments with mouse monocytes indicate that up-regulation of BER capacity in vitro correlates with enhanced cellular resistance to MMS.

**Effect of LPS on base excision repair in vivo**

An indicator of the presence of DNA lesions in vivo is the ability of a genomic DNA segment to support PCR amplification, since DNA sequences containing DNA polymerase blocking or terminating lesions will not be amplified in this assay (26–28). Conversely, removal of DNA lesions through DNA repair can enhance template integrity of genomic DNA, enabling more PCR amplification. To determine whether in vivo DNA repair was increased with LPS treatment, monocytes were treated under conditions where β-pol and BER capacity are up-regulated and then genomic DNA was isolated and examined for DNA lesions by PCR amplification (26,27). The results indicate that PCR amplification of genomic DNA was greater for LPS-treated cells as compared with untreated control cells (Fig. 4A). These results suggest that LPS treatment results in a reduction in endogenous genomic DNA lesions and the results are consistent with the observed increase in BER capacity in cell-free extracts.

**Effect of LPS treatment in a β-pol null background**

To further evaluate the idea that BER in vivo is responsible for the LPS-mediated increase in PCR amplification of genomic DNA...
Figure 2. Effect of LPS on the BER capacity of extracts from mouse monocytes and fibroblasts. Monocytes (A) and fibroblasts (B) were either control (0 h) or treated with LPS (5 µg/ml) and harvested at 2 and 8 h after treatment. Experiments were conducted as described in Materials and Methods. Photographs of autoradiograms are shown. The BER products are indicated as 51mer, the product of the overall BER reaction, and as 22mer, the intermediate product representing single nucleotide synthesis (i.e. [32P]dCMP incorporation). The bar graph in each panel illustrates the relative amount of BER product (51mer) formed.

(see for example Fig. 4A) experiments with wild-type and β-pol null fibroblasts (10) were performed. Extracts from these β-pol null cells are deficient in uracil-initiated single nucleotide or short patch BER activity in vitro (10). For wild-type fibroblasts an increase in amplification was observed after LPS treatment (Fig. 4B), reflecting less DNA damage; most of the reduction in DNA damage had occurred by 2 h LPS treatment. In contrast, LPS treatment of β-pol null cells resulted in a decrease in PCR amplification compared with that for DNA from untreated control cells (Fig. 4B). This decrease in PCR amplification, indicating more DNA damage, is consistent with some type of LPS-induced endogenous DNA damage remaining unrepaired in the β-pol null background. Overall, these results with wild-type and β-pol null cells suggest that β-pol-dependent BER is involved in preventing accumulation of genomic DNA damage after LPS treatment.

The effect of pre-treatment with LPS on the sensitivity of wild-type and β-pol null fibroblasts to MMS exposure was also examined. The experiment was conducted as a function of the period of LPS pre-treatment. Concentrations of MMS were selected to provide approximately equal MMS sensitivity in the two cell lines (Fig. 5). First, LPS pre-treatment for 18 h made wild-type cells more resistant to MMS (Fig. 5B), however, pre-treatment for 4 or 8 h did not make these cells more resistant. In contrast, LPS pre-treatment of the β-pol null cells failed to make them more resistant, but instead made the cells slightly more sensitive (Fig. 5A). These results indicate that LPS treatment of mouse fibroblasts correlates with enhanced cellular resistance to MMS and that this effect is not observed in cells lacking the β-pol-dependent base excision repair pathway.

DISCUSSION

In this study we show that LPS treatment of mouse monocytes and fibroblasts increases the cellular β-pol level and BER activity in extracts prepared from these cells. LPS treatment correlates with less cellular sensitivity to a DNA damaging agent (MMS) that leads to BER-corrected DNA lesions. LPS treatment also results in changed genomic DNA in vitro, such that the DNA is a better template for PCR amplification, thus reflecting less DNA damage. These increases in cellular MMS resistance and repair of endogenous DNA lesions appear to require the β-pol-mediated BER pathway, as β-pol null cells have neither response. These in vitro results are plausible, as recent studies have revealed that β-pol contains two of the enzymatic activities involved in uracil-initiated or abasic site-initiated single nucleotide BER (8–10). Since LPS activates monocytes and fibroblasts to produce NO (11,28–33), several kinds of LPS-induced endogenous DNA damage are expected, including base modifications and base loss (6,7). These lesions are considered to be repaired by the β-pol-dependent BER pathway (6,7).

Figure 3. Effect of LPS treatment on the sensitivity of mouse monocytes to MMS. Monocytes were pre-treated with 5 µg/ml LPS and then with MMS as described in Materials and Methods. LPS pre-treatment was in medium containing 10% (A) or 0.5% (B) fetal bovine serum. Results shown are untreated (control, ○) and LPS pre-treated (■); the data are from one of four similar experiments. The inset in each panel is a photograph illustrating the results of immunoblot analysis of β-pol protein in the indicated LPS-treated whole cell extract.
Figure 4. Effect of LPS on the templating capacity of genomic DNA from monocytes and wild-type (+/+ ) and β-pol null (−/− ) fibroblasts. Monocytes or wild-type and β-pol null fibroblasts were treated with LPS (5 µg/ml) for the indicated times and then harvested for DNA isolation. Relative amplification of a DNA fragment in monocytes (A) and in fibroblasts (B) was performed as described under Materials and Methods. Control and zero time refer to cells without LPS treatment. The inserts at the top of each panel are autoradiograms representative of PCR from duplicate plates, as indicated. The data are expressed as the mean, with bars indicating ± SD (n = 3 PCR analyses). (B) The open and hatched bars are for wild-type and β-pol null fibroblasts respectively. The decrease in amplification, relative to the control, was calculated as described under Materials and Methods. The two panels on the bottom demonstrate the linearity of PCR amplification for both the β-globin (left) and β-pol (right) genes upon increasing template concentration. This linearity supports the quantitative reliability of amplification under these reaction conditions, as described under Materials and Methods.

Figure 5. Effect of period of LPS pre-treatment on the sensitivity of mouse fibroblast to MMS. β-pol null (A) and wild-type (B) fibroblasts were pre-treated with LPS, as described under Materials and Methods, for the period indicated and then with MMS for 1 h. Cells were then washed and allowed to grow for 3 days. The data are the mean of triplicate cultures; the data are from one of two similar experiments. The value in parenthesis is the percent cells per dish relative to untreated control (100%). The shaded bars represent the greatest difference in sensitivity between wild-type and β-pol null cells.

The possibility that deregulation of β-pol could alter BER in these cell lines had been suggested by previous biochemical experiments. For example, when purified β-pol was added to extracts from untreated (i.e. control) monocyte (data not shown) and fibroblast cells (10) BER activity measured in vitro also increased. This increase in BER was to a level similar to that observed here (Fig. 2) with extracts from LPS-treated cells (data not shown), suggesting that β-pol could be rate limiting in the control cell extract and that an increase in β-pol expression could be responsible for induction of BER capacity.

Although the conditions for the cell sensitivity experiments with monocytes and fibroblasts were different, there was an overall correlation in both cases between LPS treatment and enhanced cellular resistance to MMS. Monocytes required low serum conditions for BER up-regulation and for the cellular MMS resistance effect, whereas 10% serum could be used with fibroblasts. Such differences between cell lines are not surprising, based upon our experience of cell differences in gene expression studies and could reflect differences in in vivo DNA damage/repair and in signal transduction pathways in the two cell types. In our experiments fibroblasts required a period of LPS pre-treatment in order to exhibit cellular MMS resistance. This was reminiscent of the results of Wiese et al. (34), who found a similar temporal pre-treatment effect for an adaptive increase in resistance to H2O2 in several mammalian cell lines (34).

Induction of β-pol gene expression by LPS could result from several known mechanisms. The well-known activation of the tyrosine kinase/mitogen-activated protein kinase pathway could lead to activation of transcription factors and induction of β-pol gene expression (23,35). In addition, activation of protein kinase C by LPS has been demonstrated and this could lead to
up-regulation of β-pol expression (36). It is also possible that oxidative DNA damage, secondary to LPS treatment, could trigger a DNA damage response, ultimately leading to activation of transcription factors that stimulate the β-pol promoter (23,37,38). At the present time, however, we do not know the mechanism of up-regulation of β-pol expression by LPS and further studies will be required to address this topic.

In summary, in both mouse monocytes and fibroblasts β-pol expression is up-regulated by oxidative stress-inducing agents. LPS, a strong up-regulator of β-pol expression (36). It is also possible that oxidative DNA damage, secondary to LPS treatment, could trigger a DNA damage response, ultimately leading to activation of transcription factors that stimulate the β-pol promoter (23,37,38). At the present time, however, we do not know the mechanism of up-regulation of β-pol expression by LPS and further studies will be required to address this topic.

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