Apurinic/Apyrimidinic Endonuclease 1 Regulates Inflammatory Response in Macrophages

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Abstract

The multi-functional apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) DNA repair and redox signaling protein has been shown to have a role in cancer growth and survival, however, little has been investigated concerning its role in inflammation. In this study, an APE1 redox-specific inhibitor (E3330) was used in lypopolysaccharide (LPS)-stimulated macrophages (RAW264.7). E3330 clearly suppressed secretion of inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL-6) and IL-12 and inflammatory mediators nitric oxide (NO) as well as prostaglandin E_2 (PGE_2) from the LPS-stimulated RAW264.7 cells. These data were supported by the down-regulation of the LPS-dependent expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes in the RAW264.7 cells. The effects of E3330 were mediated by the inhibition of transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) in the LPS-stimulated macrophages, both known targets of APE1. In conclusion, pharmacological inhibition of APE1 by E3330 suppresses inflammatory response in activated macrophages and can be considered as a novel therapeutic strategy for the inhibition of tumor-associated macrophages.

Keywords

Apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1 or APE1) is a multifunctional protein which functions as the DNA base excision repair protein and as a redox signaling

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factor keeping transcription factors in an active reduced state (1, 2). APE1 stimulates the DNA binding activity of a number of transcription factors involved in cancer and inflammation (*e.g.* activator protein 1 [AP-1], nuclear factor-κB [NF-κB], hypoxia-inducible transcription factor 1α [HIF-1α], cAMP response-element binding [CREB], p53 and others); thus, APE1 has been suggested as a therapeutic and chemopreventive target (1–8). Indeed, E3330, a small-molecule inhibitor of the APE1 redox domain, suppressed proliferation and migration of pancreatic cancer cells and inhibited growth of pancreatic cancer-associated endothelial cells, respectively (9–11). APE1 was significantly increased in the colon epithelium in patients with ulcerative colitis, a precursor of colon cancer (12). Moreover, APE1 modulated inflammatory responses through Toll-like receptor 2 (TLR2) by the activation of NF-κB and HIF-1α resulting in the expression of inflammatory cytokines and chemokines tumor necrosis factor-α (TNF-α) and chemokine (C-X-C motif) ligand 8 (CXCL8) in primary keratinocytes (13).

The tumor microenvironment contains stromal cells such as fibroblasts, endothelial cells, and macrophages. In spite of the importance of macrophages in the host-defense mechanism and inflammation (14, 15), the overproduction of inflammatory mediators by macrophages has also been implicated in cancer (16), whereas anti-inflammatory drugs reduce the risk of cancer (17). Further, a high density of tumor-associated macrophages correlates with poor prognosis in a majority of published studies, including those on breast, prostate, bladder, kidney, endometrial and esophageal carcinomas (18, 19). Thus, the inflammatory stimuli from macrophages promote cancer growth (20–22), whereas the reduction of macrophages results in the decrease of tumor growth (22). Macrophage activations result in the secretion of a number of different inflammatory mediators, including TNF-α, interleukin-6 (IL-6), reactive oxygen species (ROS), prostaglandin E_2 (PGE_2) and nitric oxide (NO) (23–29). Lipopolysaccharide (LPS), a constituent of the gram-negative bacterial cell wall, interacts with TLR4, which is expressed on macrophages (30, 31). The interaction between LPS and the TLR4 receptor complex activates intracellular signaling through myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways leading to the activation of transcription factors NF-κB and AP-1 and the expression of TNF-α and IL-6 (32, 33). In addition, LPS induces expression of IL-12 in macrophages through NF-κB (27, 34), and AP-1 (35). Moreover, LPS-dependent expression of cyclooxygenase-2 (COX-2), which controls production of PGE2, and inducible nitric oxide synthase (iNOS), which controls production of NO, is also regulated through NF-κB and AP-1 (36).

In the present study, the anti-inflammatory activity of E3330, a small-molecule inhibitor of APE1 redox signaling function, was investigated in LPS-challenged RAW264.7 macrophages.

Materials and Methods

Cell culture

RAW264.7 cells, a murine macrophage cell line (American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. The RAW264.7 cells were plated at a density of 2–3×10^6 and cultivated at 37°C in a humidified atmosphere containing 5% CO_2. For all the experiments, the cells were grown to 80–90% confluence. The RAW264.7 cells were stimulated with 1 μg/ml LPS (Sigma, St. Louis, MO, USA). The cells were incubated with or without E3330 in the serum free medium for 1 hour before exposure to LPS. E3330 was synthesized as previously published (4, 37, 38), and used as described in the individual experiments.

Expression of pro-inflammatory mediators in RAW264.7 cells

The RAW264.7 cells were preincubated with 0–25 μg/ml E3330 for 24 hours followed by an additional 24 hours' incubation with LPS (1 μg/ml). The levels of TNF-α, IL-6, IL-12p40 (Biolegend, San Diego, CA, USA) and PGE2 (R&D Systems, Minneapolis, MN, USA) were determined by ELISA; the release of NO was determined by Griess reagent (Sigma) as previously described (39).

Preparation of whole-cell and nuclear extracts

After E3330 and LPS treatments (the whole-cell and nuclear extracts were prepared as described previously (39). The protein concentration in the whole-cell and nuclear extracts was determined according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

Western blot analysis was performed as previously described (39), on RAW264.7 cells pretreated with E3330 (0–25 μg/ml) for 24 hours followed by LPS (1 μg/ml) for 24 hours. Briefly, the total cell lysates were separated by SDS–PAGE and were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin in TBS-Tween 20 solution and was further incubated with the corresponding antibody: COX-2 (BD Biosciences, San Jose, CA, USA), iNOS and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reactive bands were visualized with horseradish peroxidase (HRP)-coupled secondary antibody *via* an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's procedures.

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts from RAW264.7 cells pretreated with E3330 (0–25 μg/ml) for 24 hours followed by LPS (1 μg/ml) for 30 minutes were used. Oligonucleotide probes containing consensus sequences for AP-1-and NF-κB-binding sites were purchased from Promega (Madison, WI, USA). EMSA for AP-1 and NF-κB was performed with a ^{32}P-AP-1 or ^{32}P-NF-κB probe in gel shift binding buffer with 5 μg of nuclear protein as previously described (39). The specificity was confirmed by competitive EMSA with cold AP-1 or NF-κB oligonucleotide or unrelated DNA (URL).

Statistical analysis

Data are presented as mean±S.D and were analyzed by Student *t*-test. Results were considered significant if $p \leq 0.05$.

Results

Effect of E3330 on LPS-dependent production of pro-inflammatory cytokines

As expected, LPS stimulation markedly induced the production of TNF-α from the RAW264.7 cells, whereas pretreatment with E3330 at doses 12.5 and 25 μg/ml significantly suppressed TNF-α production from these macrophage cells (Figure 1A). In addition, E3330 (6.25–25 μg/ml) also significantly suppressed IL-6 production from LPS-stimulated RAW264.7 cells (Figure 1B). Moreover, LPS-dependent production of IL-12 in macrophages was also markedly suppressed by E3330 in a dose-dependent manner (Figure 1C).

Effect of E3330 on LPS-dependent secretion of PGE2 and NO and on the regulation of expression of COX-2 and iNOS

E3330 treatment markedly decreased the secretion of the LPS-induced inflammatory mediators PGE_2 (Figure 2A) and NO (Figure 2B) in a dose-dependent manner. Because the production of PGE_2 and NO is controlled by COX-2 and iNOS, respectively, the expression of COX-2 and iNOS was evaluated by Western blot. As shown in Figure 2C, LPS induced the expression of COX-2 in the RAW264.7 cells, whereas E3330 pretreatment suppressed this LPS-dependent expression. In addition, the LPS-induced expression of iNOS was also markedly reduced by the E3330 in the macrophages (Figure 2D).

Effect of E3330 on LPS-inducible NF-κB and AP-1 activation

NF-κB and AP-1 DNA-binding activity was evaluated in nuclear extracts by gel shift analysis. As shown in Figure 3A, LPS markedly induced the binding activity of nuclear extracts to the NF-κB DNA consensus sequence, whereas pretreatment of the macrophages for 24 hours with E3330 (12.5 and 25 μg/ml) suppressed the LPS-dependent increase of NF-κB binding. As expected, the binding activity of AP-1 in nuclear extracts was also induced by the LPS treatment, and E3330 pretreatment suppressed this LPS-dependent binding activity of AP-1 in the RAW264.7 cells (Figure 3B).

Discussion

The inhibition of APE1 redox signaling function by the APE1-specific small molecule E3330 suppressed the LPS-dependent production of pro-inflammatory cytokines, TNF-α, IL-6, and IL-12, and inflammatory mediators, NO and PGE_2, in the murine macrophages RAW264.7 cells. The inhibition of secretion of NO and PGE_2 by E3330 was mediated through the down-regulation of expression of iNOS and COX-2, respectively. In addition, E3330 inhibited the LPS-dependent induction of NF-κB and AP-1. Mechanistically, E3330 inhibits or blocks the redox function of APE1 resulting in the cysteine residues located in the DNA binding domain of p50 (NF-κB) and Fos and Jun (AP-1) not being converted from an oxidized to a reduced status. This results in the inability of NF-κB and AP-1 to bind to their respective DNA target sequences (Figure 4) (40, 41).

The present study was in congruence with original reports demonstrating the suppression of LPS-dependent production of TNF-α by E3330 through the inhibition of NF-κB in macrophages and monocytes (42, 43). The inhibition of TNF-α by E3330 has also been associated with the protection against endotoxin-mediated hepatitis and alcoholic liver injury (44, 45). However, the data presented here demonstrates, for the first time, that the inhibition of NF-κB and AP-1 by E3330 can directly suppress production of IL-6, IL-12, PGE_2 and NO and down-regulate expression of COX-2 and iNOS in activated macrophages.

APE1 has been the focus of a number of studies, from use as a diagnostic aid in cancer screening (46) to a suitable target for the prevention or treatment of cancer (1). However, more intensive studies have focused on both repair and redox functions of APE1 as a novel therapeutic target in cancer (3–5). Although recent studies have demonstrated a direct effect of E3330 on a variety of cancer cells (1, 9, 37, 38–34, 47–51), the inhibition of the inflammatory response by E3330 in activated macrophages is of particular interest.

The inhibition of NF-κB and AP-1 signaling in activated macrophages by E3330 could have a potential therapeutic effect as NF-κB and AP-1 are necessary for the inflammatory response leading to the production of TNF-α, IL-6, IL-12, NO and PGE_2 and other inflammatory mediators in these cells (23–29). As mentioned above, a link between activated macrophages and cancer has been demonstrated (18, 20–22), and a recent study further confirmed that an increased number of tumor-associated macrophages was strongly

associated with shortened survival of cancer patients (19). Although the depletion of macrophages with clodronate inhibited tumor recovery after irradiation (52), this strategy would also deplete other non-tumor-associated macrophages and would therefore interrupt the homeostatic function of macrophages in the gut, liver, lung and spleen in humans (53). Therefore, direct targeting of tumor-associated macrophages is a more plausible strategy. Blocking TNF-α with TNF-α receptor or inhibiting macrophage recruitment by macrophage antibodies inhibited tumor regrowth after tumor irradiation, respectively (52, 54). Another possible strategy would be the inhibition of secretion of pro-inflammatory cytokines and mediators from macrophages by E3330 as presented in the current study.

In summary, E3330 exerts an anti-inflammatory effect by blocking the ability of APE1 to convert NF-κB and AP-1 from an oxidized to a reduced state. These two transcription factors have been implicated in signaling in LPS-activated macrophages, Thus E3330 treatment results in the suppression of the production of pro-inflammatory cytokines and mediators. The inflammatory response in LPS-stimulated macrophages controlled by APE1 and has a potential clinical applicability to affect the tumor microenvironment as having direct effects on tumor cells. The demonstration of an anti-inflammatory activity of E3330 provides further proof-of-concept data promoting the development of E3330 as an anti-inflammatory agent for the inhibition of the detrimental function of tumor-associated macrophages in cancer treatment. Furthermore, it continues to illustrate the important function of APE1 redox signaling in the control of the tumor microenvironment.

Acknowledgments

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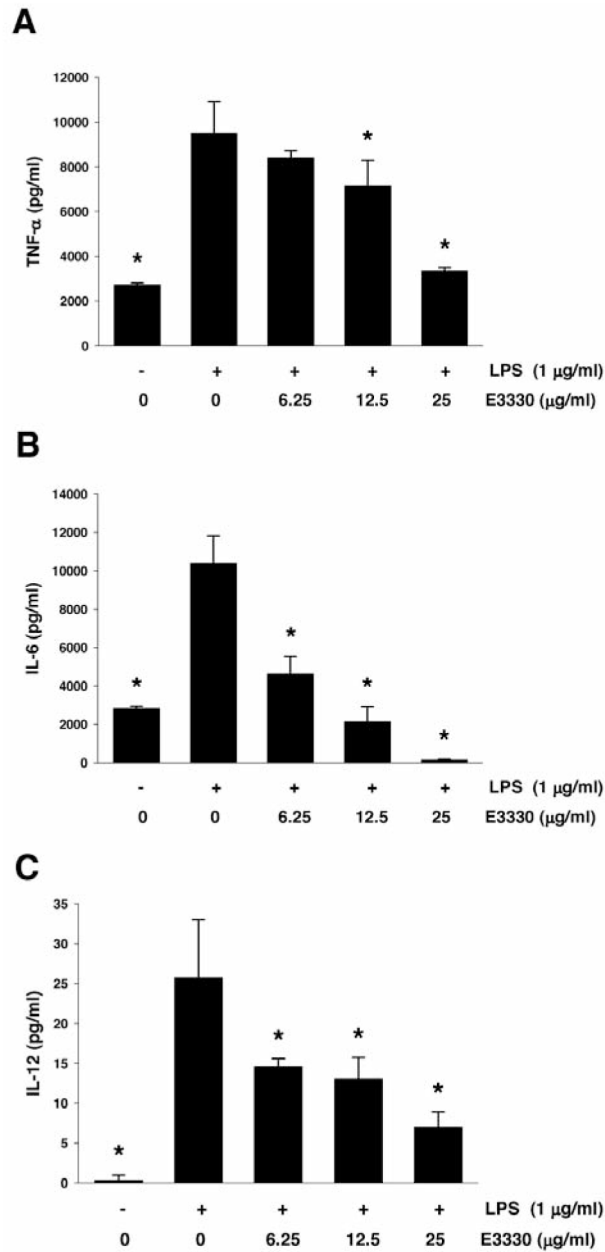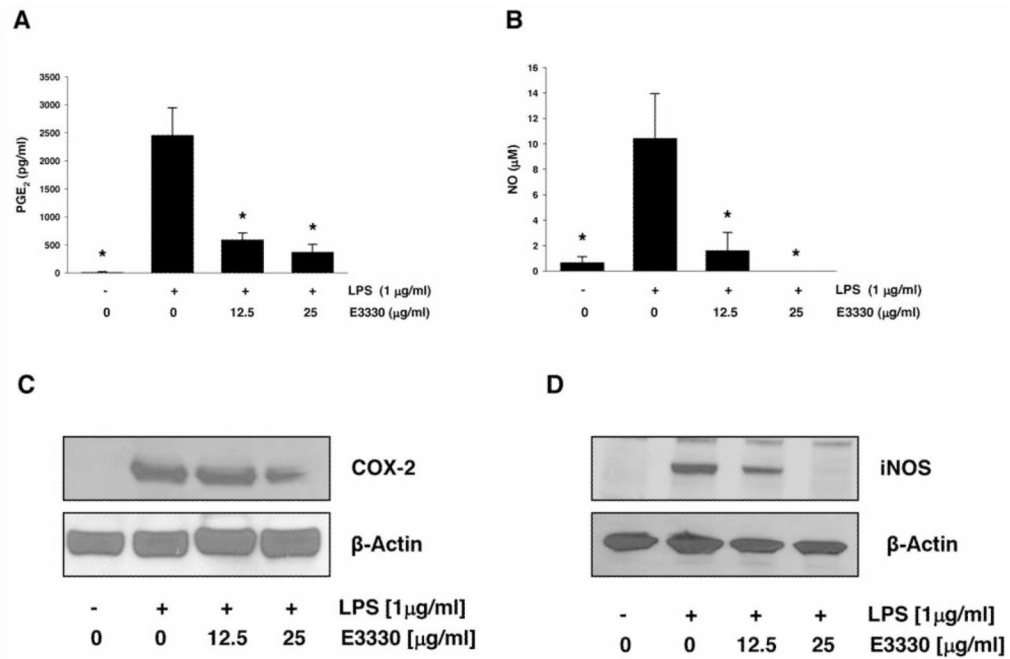
Figure 1.
Effect of E3330 on LPS-induced production of pro-inflammatory cytokines in RAW264.7 cells. (A) TNF-α, (B) IL-6 (C) IL-12 production in cell culture media from RAW264.7 cells pretreated with E3330 for 24 hours followed by LPS for an additional 24 hours. Each value is the mean±S.D. of two independent experiments, repeated minimally twice. *p<0.05 control vs. LPS alone, *p<0.05 LPS vs. LPS plus E3330.

Figure 2.
Effect of E3330 on LPS-induced PGE$_2$, NO, COX-2 and iNOS expression in RAW264.7 cells. RAW264.7 cells were pretreated with E3330 for 24 hours followed by LPS for 24 hours. (A) PGE$_2$ and (B) NO in cell culture media. Each value is the mean±S.D. of two independent experiments, repeated minimally twice. $p<0.05$ control vs. LPS alone, *$p<0.05$ LPS vs. LPS plus E3330. (C) COX-2 and (D) iNOS determined in lysates by Western blot analysis. The equal protein loading was verified with anti-β-actin antibody. The results are representative of three separate experiments.
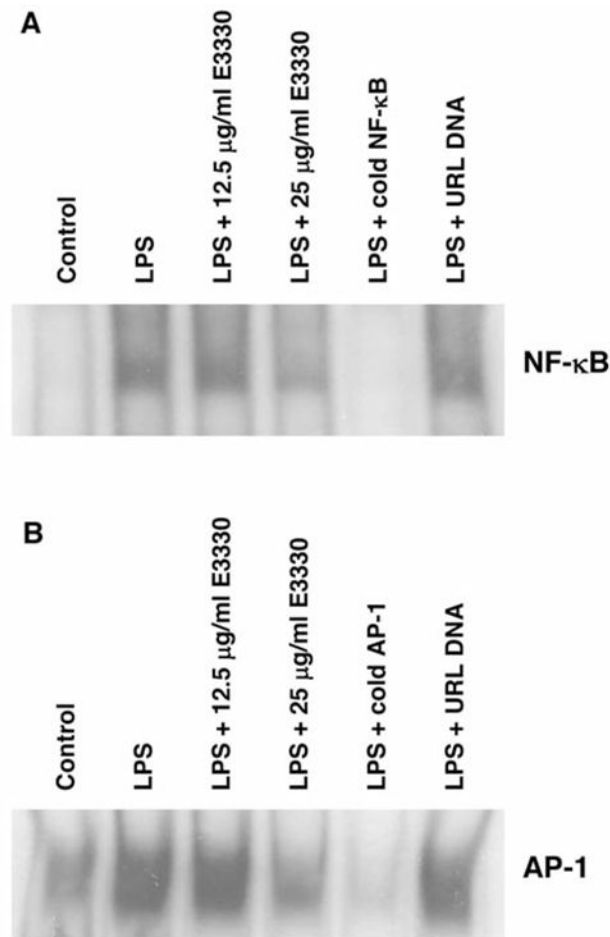
Figure 3.
Effect of E3330 on NF-κB and AP-1 DNA-binding. RAW264.7 cells were pretreated with E3330 for 24 hours followed by the LPS (1 μg/ml) treatment for 30 min. Nuclear extracts were subjected to gel shift analysis with (A) [^{32}P]-labeled NF-κB probe or [^{32}P]-labeled AP-1 probe. The specificity was confirmed by the competitive gel shift in LPS-stimulated cells with cold NF-κB or AP-1 or unrelated DNA (URL). The data are representative of three separate experiments.
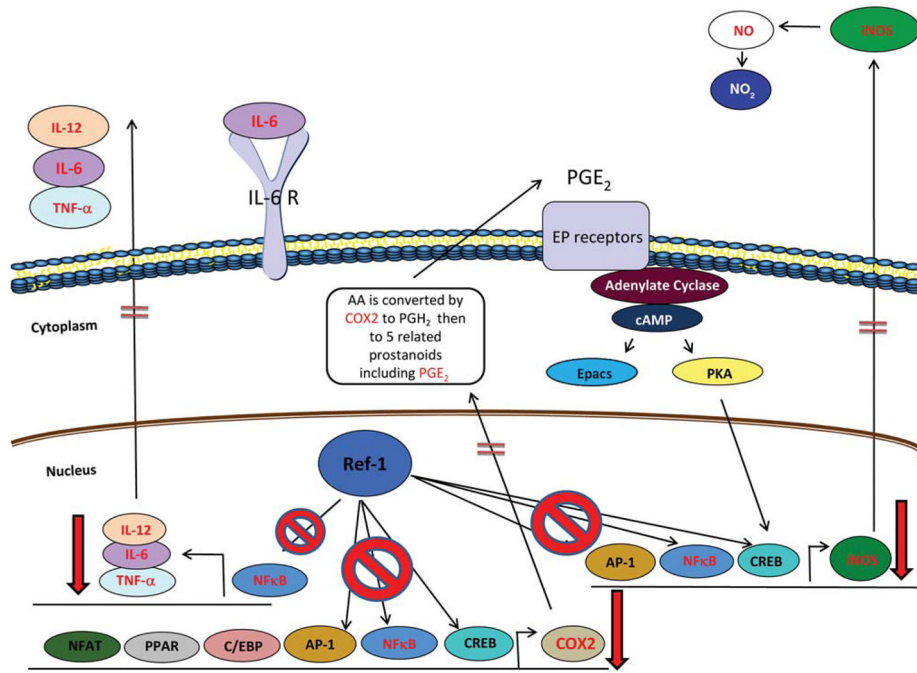
Figure 4.
Schematic illustration of the relationship of affected molecules and pathways following inhibition of Ref-1 (APE1) redox function with E3330. Blocking Ref-1 with the small molecule Ref-1 redox inhibitor E3330 inhibits NF-κB function and most likely other transcription factors that are downstream of Ref-1 such as AP-1 and CREB leading to a decrease in synthesis of COX2, IL-12, IL-6, TNF-α and iNOS. Decreased levels of iNOS result in decreased NO produced. Decreased production of COX2 leads to fewer AA being converted by COX2 and eventually to PGE_2 which could result in a signaling cascade decrease that further suppresses iNOS production via CREB suppression. NFAT, Nuclear factor of activated T-cells; PPAR, peroxisome proliferator-activated receptor; CREB, cAMP response element-binding; PR, prostagladin receptors; C/EBP, CCAAT-enhancer binding proteins. The red lettering signifies molecules measured in the present study.