Oxidative stress activates NLRP3 inflammasomes in ARPE-19 cells—Implications for age-related macular degeneration (AMD)

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Abstract

Oxidative stress and inflammation are known to be associated with age-related macular degeneration (AMD). Retinal pigment epithelial (RPE) cells play the principal role in the immune defense of macula, and their dysfunction is a crucial event leading to clinically relevant changes seen in AMD. In the present study, we have examined the ability of oxidative stress to activate inflammasome signaling in the human ARPE-19 cells by adding the lipid peroxidation end product 4-hydroxynonenal (HNE) to cell cultures pre-treated or not treated with the endotoxin, LPS. Our results indicate that LPS and HNE significantly increased the production of IL-6 and IL-18, respectively. LPS treatment preceding HNE induced an even greater increase in the production of IL-18 than HNE alone. In addition to IL-18, HNE significantly increased the production of IL-1β. The productions of IL-1β and IL-18 were reduced in the cell cultures pre-treated with the Caspase-1 inhibitor, PCR analysis revealed that HNE induced an over 5-fold increase in the amount of NLRP3 mRNA compared to control cells; LPS had no effect. In conclusion, our present data suggest that oxidative stress can activate NLRP3 inflammasomes in RPE cells which occupy central stage in the pathogenesis of AMD.

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1. Introduction

Aging predisposes cells to oxidative stress, a condition where the amount of intracellular oxidizing agents becomes increased with a concurrent decline in the cell’s defensive systems [1]. Oxidative stress and inflammation are known to be associated with age-related macular degeneration (AMD) which is the leading cause of blindness in the elderly all around the world [2]. AMD is a neurodegenerative disease with a multifactorial aetiology, primarily attributable to morphological and functional abnormalities in the retinal pigment epithelial (RPE) cells [3]. The disease is characterized by a progressive loss of central vision because of degenerative and neovascular changes in the macula, the highly specialized region of the central retina responsible for fine and color vision. Due to increasing life expectancy, the number of patients is continuously increasing, and AMD is becoming a major public health burden.

Retinal pigment epithelium (RPE) is a single layer of postmitotic cells which plays a key role in the maintenance of the overlying photoreceptor layer. RPE cells regulate the transport of nutrients and waste products to and from the retina, and contribute to outer segment renewal by ingesting and degrading the spent tips of photoreceptor outer segments (POS) [4]. The RPE layer protects the outer retina from excessive high-energy light and light-generated reactive oxygen species, and maintains retinal homeostasis through the release of diffusible factors. RPE cells also play a principal role in the immune defense of macula. Aging in its various manifestations induces a vicious circle of stress, inflammation and lipofuscin accumulation in RPE cells [5,6]. Dysfunction of RPE cells is an early and crucial event in the onset of disease, leading to the clinically relevant changes encountered in AMD [7].

The innate immune system monitors cellular stress factors through pattern recognition receptors (PRRs). PRRs are receptors which recognize highly conserved molecular structures called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [8]. Toll-like receptor (TLR), Nod-like receptor (NLR), C-type lectin receptor (CLR), and the virus-specific RIG-I-like receptor (RLR) systems are the major PRR pathways in cells [8]. NLR proteins are the sensing components
of inflammasome complexes the best known of which are NLRP3 inflammasomes. If a danger signal is encountered by NLRP3 (also known as NALP3, CATERPILLER, CIA51, Cryopyrin or PYPAF1) then this triggers the assembly of multimeric structure including NLRP, ASC (an apoptosis associated speck-like protein containing CARD and PYD domains), and caspase-1 components [9–11]. The assembly of the inflammasome results in the processing and activation of caspase-1 enzyme which subsequently activates the pro-inflammatory cytokines, IL-1β and IL-18 [9]. While the cytokines can be produced through TLR-NF-κB signaling, they remain as inactive intracellular precursors until they are activated by caspase-1 [9,11].

The present study examined the ability of oxidative stress to activate inflammasome signaling in ARPE-19 cells. These results provide convincing evidence that oxidative stress may be a potential inducer of inflammasome activation in human RPE cells.

2. Materials and methods

2.1. Cells and stimulations

ARPE-19 cells were purchased from the American Type Culture Collection (ATCC). The cells were grown in a humidified 10% CO₂ atmosphere at 37 °C in Dulbecco’s MEM/Nut Mix F-12 (1:1) medium (Life Technologies, Paisley, UK) containing 10% inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin (Cambrex, Charles City, IA, USA), 100 µg/ml streptomycin (Cambrex), and 2 mM L-glutamine (Life Technologies). Confluent cell cultures were washed with serum-free DMEM/F12 medium (with supplements) and stimulated with LPS (10 µg/ml; Sigma–Aldrich Finland, Helsinki, Finland) in serum-free medium for 24 h in a humidified 10% CO₂ atmosphere at 37 °C.

2.2. ELISA measurements

Medium samples were collected 24 h after the induction of oxidative stress and stored at −70 °C until analyzed. The concentrations of soluble pro-inflammatory cytokines IL-18, IL-1β, and IL-6, were measured in duplicate using commercial ELISA reagents (OptEIA® sets obtained from BD Pharmingen). All ELISA measurements were performed according to the manufacturer’s protocols.

2.3. Real-time PCR

Total RNA was isolated from the cells using NucleoSpin® RNA/Protein columns (Macherey-Nagel GmbH & Co., Düren, Germany) as instructed by the manufacturer. Reverse transcription of RNA to cDNA was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen by Life Technologies Europe BV). The amount of human GAPDH, NLRP1, and NLRP3 RNAs was assayed by the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems by Life Technologies Europe BV) using SYBR® Green chemistry (Applied Biosystems). The thermocycling program was 40 cycles of 95 °C for 15 s and 60 °C for 60 s with an initial cycle of 95 °C for 10 min. The melting curve analysis was performed over the range of 60–95 °C. Real-time PCR was duplicated for each of the four parallel cDNA samples, and the samples from two independent experiments were measured. cDNA was semi-quantified, and analyzed with the ΔΔCt (change in cycle threshold, Ct) method [12]. The primer sequences were as follows: GAPDH, forward 5′-CGAGATCCCTCTACTAACAA-3′ and reverse 5′-GTTGAATAGGGACTGTTG-3′; NLRP1, forward 5′-CCAGAAACTGAGGAGCCTG-3′ and reverse 5′-TACACCTATGAAAGGTCTCAGG-3′ (both primer pairs designed using Primer3 Output program); NLRP3, forward 5′-GCATATCACAGTGGGATTC-3′ and 5′-GATCTGGCCTGCAGTCAAC-3′ [10]. The primers were ordered from TAG Copenhagen (Copenhagen, Denmark).

2.4. Statistical analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical differences between groups were assessed using the Mann–Whitney U-test. P-values of 0.05 or less were considered significant.

3. Results

3.1. LPS but not HNE activates the NF-κB pathway

The activation of NF-κB signaling cascade by exogenous or endogenous signals produces the pro-forms of IL-1β and IL-18 but the cytokines are not secreted unless cleaved by the inflammasome-associated caspase-1 [11]. We have previously shown that LPS is suitable for the priming process. It enhances the DNA binding activity of NF-κB in ARPE-19 cells, with a concomitant increase in IL-6 production [13]. On the other hand, our previous results also showed that HNE does not increase the DNA binding activity of NF-κB [14]. In the present study, significant production of IL-6 was evoked by LPS but not by HNE (Fig. 1). Moreover, Fig. 2 shows that priming of the cells with LPS clearly increased the IL-18 production only after the exposure to HNE. LPS alone showed a small increase in the release of IL-18 but the increase was not significant when compared to non-stimulated controls (Fig. 2). It was also almost four times lower than the IL-18 released by HNE alone (6 pg/ml vs. 22 pg/ml).

3.2. HNE induces inflammasome signaling

In order to demonstrate the activity of the inflammasome complex, ARPE-19 cells were treated with LPS and HNE, and the cytokines IL-1β and IL-18 were determined from the cell culture medium. Our results show that HNE but not LPS induced a statistically significant increase in the production of IL-18 (Fig. 2).
Moreover, the concentration of IL-18 released by HNE was almost six times higher with than without the LPS priming (127 pg/ml vs. 22 pg/ml). HNE also significantly increased the secretion of IL-1β into the cell culture medium (Fig. 3). It is known that IL-1β tends to act locally via paracrine signaling, i.e. it is used by the cells in the immediate vicinity [15]. Therefore, we blocked the cell surface receptors using an IL-1 receptor antagonist (IL-1RA) which prevented the IL-1β-mediated stimulation of the cells (data not shown).

The productions of both IL-1β and IL-18 were clearly reduced when the function of caspase-1 was blocked with a caspase-1 inhibitor (Figs. 2 and 3). The inhibition response was specific since the cytokine production was not significantly reduced with the pre-treatment of DMSO, the solvent used to dissolve the inhibitor (data not shown).

3.3. HNE significantly increases the NLRP3 mRNA level

The inflammasome component which is involved in recognizing non-pathogenic stress signals is usually NLRP1 or NLRP3, and both inflammasome types are able to process and activate the pro-inflammatory cytokines IL-1β and IL-18 [16]. Real-time PCR analyses were performed in order to study the level of mRNA encoding NLRP1 or NLRP3 in ARPE-19 cells. There was a significant increase in the amount of NLRP3 mRNA in the cells exposed to HNE but not in those treated with LPS (Fig. 4). Neither HNE nor LPS elevated the mRNA level of NLRP1 (Fig. 4). All the samples displayed a similar mRNA level of the endogenous housekeeping protein GAPDH (Fig. 4).

4. Discussion

RPE cells are constantly exposed to oxidative stress and lipid peroxidation. 4-Hydroxy-2-nonenal (HNE) is a highly reactive end-product of lipid peroxidation reaction which is a common event in aging cells [17]. It has shown to be an important marker in several inflammation-related disorders, such as cardiovascular diseases, stroke, Alzheimer’s disease, arthritis, and asthma [17]. In RPE cells, HNE is considered to be a causative factor in detrimental lipofuscin generation, increased cellular stress responses and cell death [14,18,19]. There are strong clinical indications obtained from AMD patients that excessive lipofuscin accumulation increases RPE damage and promotes the AMD process [20]. Our present results show that HNE significantly increased the production of IL-18 by human ARPE-19 cells (Fig. 2). The increase in the IL-18 production by HNE alone is not surprising since there can be small amounts of pro-IL-18 constantly present in the cells [21]. The production of IL-18 increased even more dramatically when the cells were pre-treated with LPS (Fig. 2). Interestingly, LPS alone did not release significant amounts of IL-18 suggesting that it is not able to activate inflammasome signaling similarly to HNE. Since a strong increase in the IL-18 release was seen after the stimulation with both LPS and HNE, LPS most probably produced the pro-form of the cytokine which thereafter was maturated by HNE. HNE also induced the secretion of IL-1β (Fig. 3) although the level was low probably due to tight regulation of the cytokine [22]. Despite of low concentrations, our IL-1β response to HNE was statistically significant and reproducible. Moreover, the profile of the IL-1β data was identical to that seen for IL-18. Our results fit well in the classical function of
The growth wells transcription produced complex inflammasome in Fig. 32 A. HMGB1 the masome that activates forms the inflammasome important triggers LPS pro-forms renewal monocytic PGN IL-1 inflammasome-dependent cytokines. In the present study, the production of IL-6 after stimulation with LPS (Fig. 1) indicated that our endotoxin preparation was able to activate the NF-κB pathway. The inflammasome activation was most probably not elicited by MDP since no significant increase in the production of IL-18 was visible upon stimulation with LPS. LPS did not increase the amount of NLRP3 mRNA either (Fig. 4). We have also determined the NLRP3 protein from our samples, and found out that LPS, indeed, stimulates the production of NLRP3, just as in myeloid cells (data not shown). The reason for the absence of mRNA in the LPS-stimulated samples is most probably different kinetics upon LPS and HNE stimulations. It makes perfectly sense that also LPS is capable of inducing the production of NLRP3. However, the activation of NLRP3 by LPS may be anti-inflammatory instead of inflammatory [10].

To date, over twenty NLRs have been identified in the human cells. NLRP1, NLRP3, and NLRP4 of which are capable of activating caspase-1 [26,27]. NLRP1 and NLRP4 are known to respond mainly to bacterial toxins, whereas NLRP3 responds to a broader variety of signals. Oxidative stress has been closely linked to the activation of inflammasomes having NLRP3 as their NLR component [28]. In fact, it has been shown that all known NLRP3 activators generate reactive oxygen species (ROS) [29,30]. Our data is well in line with this concept since HNE significantly increased the amount of NLRP3 mRNA but not that of NLRP1 (Fig. 4). RPE cells are exposed inflammasome pathway. First, LPS upregulates the production of the pro-forms of IL-1β and IL-18, and thereafter oxidative stress triggers the inflammasome cascade. The functional inflammasome complex results in the activation of caspase-1 enzyme which then cleaves and thereby activates the cytokines into their secreted forms [11].

LPS is not the principal agent threatening RPE cells in AMD. The endotoxin was used in the present study to activate the NF-κB pathway which can be activated in vivo by various other factors. These include fragments from the extracellular matrix, e.g. hyaluronan and fibronectin, and stress-related alarmins, e.g. HSP60 and HMGB1 [23,24]. Moreover, TLR4 has been shown to participate in the renewal of photoreceptor outer segments (POS) which is an important task conducted by RPE cells [4]. A study with human primary monocytes postulated that LPS could activate the inflammasome also by itself [10]. This hypothesis is controversial since it has also been proposed that the bacterial peptidoglycans (PGN) present in endotoxin preparations would be responsible for the inflammasome activation instead of LPS [25]. Martinon et al. used the monocytic cell line THP-1 to demonstrate that it is actually the PGN degradation product muramyl dipeptide (MDP) which activates the inflammasome through NLRPs [25]. It is also known that MDP activates NF-κB through the NOD2 protein [25]. Therefore, MDP could induce both the translation and the secretion of inflammasome-dependent cytokines. In the present study, the production of IL-6 after stimulation with LPS (Fig. 1) indicated that our endotoxin preparation was able to activate the NF-κB pathway. The inflammasome activation was most probably not elicited by MDP since no significant increase in the production of IL-18 was visible upon stimulation with LPS. LPS did not increase the amount of NLRP3 mRNA either (Fig. 4). We have also determined the NLRP3 protein from our samples, and found out that LPS, indeed, stimulates the production of NLRP3, just as in myeloid cells (data not shown). The reason for the absence of mRNA in the LPS-stimulated samples is most probably different kinetics upon LPS and HNE stimulations. It makes perfectly sense that also LPS is capable of inducing the production of NLRP3. However, the activation of NLRP3 by LPS may be anti-inflammatory instead of inflammatory [10].

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In Fig. 4, we investigated the influence of LPS and HNE on the expression of NLRP1 and NLRP3 in ARPE-19 cells. The cells were stimulated for 24 h with HNE (30 μM) in four parallel wells on 12-well plates. Treatment of the cells with IL-1RA at the concentration of 100 ng/ml preceded the stimulation with HNE. Non-stimulated cells served as controls. The results are representative of two independent experiments, and indicate mean 2−[ΔΔCT] values ± SEM. GAPDH served as an endogenous control.

In Fig. 5, we tested the effects of LPS on the expression of different cytokines in RPE cells. LPS induced the expression of IL-1β, IL-6, and TNF-α. The expression of IL-1β was increased significantly by LPS stimulation. LPS stimulation also induced the expression of IL-6 and TNF-α, but the increase was not statistically significant. The expression of IL-1RA, a negative regulator of IL-1β, was not affected by LPS stimulation. The expression of IL-18 was not affected by LPS stimulation. No significant changes were observed in the expression of the pro-inflammatory cytokines IL-8 and TNF-α, which were not affected by LPS stimulation. The expression of the anti-inflammatory cytokine TGF-β was not affected by LPS stimulation. The expression of the pro-inflammatory cytokine IL-1β was increased significantly by LPS stimulation. IL-1β is a central transcription factor regulating the expression of a multitude of genes mediating stress responses, cell growth, cell survival, and apoptosis [37]. Several gene products are produced through the activation of NF-κB, e.g. the pro-inflammatory cytokines IL-6 and TNF-α, as well as the pro-form of IL-1β. Abbreviations: AMD, age-related macular degeneration; IL, interleukin; NF-κB, nuclear factor kappa B; RPE, retinal pigment epithelial cell; TLR, toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
to severe oxidative stress because of their high oxygen consumption, high levels of polyunsaturated lipids, and the long periods of exposure to light [5]. In addition to direct activation of inflammation through NLRP3, oxidative stress contributes to the progression of AMD in several other ways. We have summarized the danger signals encountered by RPE cells, and the inflammation response involved with the pathogenesis of AMD in Fig. 5. For example, oxidative stress can regulate also the expression of complement factor H (CFH): a polymorphic variant of which is strongly associated with AMD [31]. The regulation occurs via the acetylation of transcription factor FOXO3, and once acetylated, this factor is no longer capable of preventing NF-kB signaling [32]. Moreover, oxidative stress evokes changes in the lysosomes of RPE cells which then lose their ability to ingest photoreceptor outer segment (POS) material resulting in the accumulation of lipofuscin [33] and lipofuscin itself has been shown to increase oxidative stress and protein damage within the cells [34].

In this study, we have investigated the molecular mechanisms involved in the AMD-associated inflammation. AMD is divided to early and late, as well as to atrophic and exudative degeneration categories [35]. According to our results, the lipid peroxidation end-product HNE significantly increased the amount of NLRP3-specific mRNA in ARPE-19 cells. HNE-treated cells also produced amounts of the pro-inflammatory cytokines IL-1β and IL-18, whereas their production was reduced in the presence of a caspase-1 inhibitor. Pre-treatment of the cells with LPS elevated the production of IL-1β and IL-18, indicating that LPS enhanced the production of the cytokine precursors. Here, we show for the first time, evidence that oxidative stress can activate NLRP3 inflammasome signaling in RPE cells which occupy center stage in the pathogenesis of AMD.

Conflict of interest

Authors do not have any conflicts of interest.

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