

Lycopene inhibits IL-6 expression in cerulein-stimulated pancreatic acinar cells

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Abstract Reactive oxygen species (ROS) are known to be involved in the pathogenesis of acute and chronic pancreatitis. The cholecystokinin (CCK) analog cerulein causes pathophysiological, morphological, and biochemical events similar to those observed in human acute pancreatitis. The oxidant-sensitive transcription factor NF- κ B plays a critical role in the development of cerulein pancreatitis by regulating the expression of pro-inflammatory cytokines in the pancreas. Lycopene has an anti-oxidant effect in various cells. In the present study, we investigated whether cerulein induces NF- κ B activation and IL-6 expression in pancreatic acinar cells and whether lycopene inhibits these events. NF- κ B-DNA-binding activity was determined by electrophoretic mobility shift assay, and mRNA expression was analyzed by reverse transcription–polymerase chain reaction (RT–PCR) and real-time RT–PCR analyses. The IL-6 concentration in the medium was determined by enzyme-linked immunosorbent assay. Our results showed that cerulein induced IL-6 expression in a time-dependent manner. NF- κ B-DNA-binding activity and intracellular levels of ROS in pancreatic acinar cells were increased by cerulein. Lycopene inhibited the cerulein-induced increase in intracellular ROS, NF- κ B activation, and IL-6 expression in pancreatic acinar cells in a dose-dependent manner. In conclusion, lycopene may be beneficial in the prevention and/or treatment of acute pancreatitis by inhibiting the activation of NF- κ B and the expression of inflammatory cytokines through reduction in intracellular levels of ROS in pancreatic acinar cells.

Keywords Lycopene · IL-6 · NF- κ B · Cerulein · Pancreatic acinar cells

Introduction

Acute pancreatitis is an inflammatory disease associated with the activation and release of pancreatic enzymes in the interstitium, leading to autodigestion of the pancreas and multiple organ dysfunction after their release into the systemic circulation, and with increased production and release of cytokines, which can ultimately lead to deleterious local and systemic effects [1–3]. Similar symptoms are observed in experimental animal models treated with cerulein, a cholecystokinin (CCK) analog. Supramaximal doses of cerulein result in experimental pancreatitis, which is characterized by dysregulation of the production and secretion of digestive enzymes, in particular inhibition of pancreatic secretions, an elevation in serum enzyme levels, cytoplasmic vacuolization, death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas [4, 5].

Oxidative stress is regarded a major pathogenic factor in acute pancreatitis [6]. In human acute pancreatitis, increased levels of lipid peroxide in the bile or pancreatic tissue and subnormal levels of antioxidant vitamins in the blood were reported [7–9]. Cerulein produces large amounts of reactive oxygen species (ROS), activates the oxidant-sensitive nuclear transcription factor NF- κ B, and induces cytokine expression in vitro in freshly isolated pancreatic acinar cells in the absence of inflammatory cells [10].

Pancreatic inflammation is initiated by local production of mediators such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-8 (IL-8) [11]. IL-6 is a pro-inflammatory cytokine that is produced by a wide range of cells including monocytes/macrophages, endothelial cells,

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fibroblasts, and smooth muscle cells in response to stimulation by endotoxin, IL-1 β , and TNF- α [12]. Elevated levels of IL-6 have been described in a number of acute conditions such as burns, major surgery, and sepsis [13]. IL-6 levels are increased in patients with acute pancreatitis and correlate with disease severity [14].

Nuclear factor- κ B (NF- κ B) is a pleiotropic transcription factor that regulates the activation of a wide variety of genes that respond to immune or inflammatory signals, including cytokine expression [15, 16]. NF- κ B represents a family of proteins sharing the Rel homology domain, which bind to DNA as homodimers or heterodimers. NF- κ B activates a multitude of cellular stress-related and inflammatory genes including IL-6. NF- κ B is kept silent in the cytoplasm via interaction with inhibitory proteins of the I κ B family and is activated by a variety of agents, including cytokines, mitogens, and ROS [17].

Lycopene, the most abundant carotenoid and responsible for the red color of tomatoes, is known to be an effective singlet oxygen quencher [18] and a potent antioxidant [19]. Epidemiological studies have indicated that elevated ingestion of tomatoes and tomato products containing lycopene is associated with a decreased risk of chronic diseases and cancers, especially pancreatic cancer [20, 21]. In addition, lycopene has anti-proliferation and pro-differentiation activities in various types of cancer cells including breast cancer, lung cancer, and endometrial cancer [22].

In the present study, we investigated whether the antioxidant lycopene inhibits NF- κ B activation and IL-6 expression in cerulein-stimulated pancreatic acinar cells.

Materials and methods

Isolation of pancreatic acinar cells

Pancreatic acinar cells were freshly isolated from Male Sprague–Dawley rats (200–220 g) by enzymatic digestion as previously described [10]. The acinar cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Lonza, Walkersville, MD, USA), 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Company, St. Louis, Missouri), and antibiotics (GIBCO). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cell viability was determined by the trypan blue dye exclusion test; more than 95% of the cells survived after 24-h culture.

Experimental protocol

The cells were stimulated with various concentrations of cerulein (10⁻⁹–10⁻⁷ M) for 3 h (for mRNA expression,

Fig. 1a) or with 10⁻⁸ M cerulein for the indicated time periods (for mRNA expression, Fig. 1b; IL-6 levels in the medium, Fig. 1c; and NF- κ B activation, Fig. 3a). Cells were treated with lycopene (2 or 5 μ mol/l) for 2 h prior to cerulein stimulation for 3 h (for mRNA expression, Fig. 2a), 6 h (for IL-6 levels in the medium, Fig. 2b), 30 min (for NF- κ B activation, Fig. 3b), or 15 min (for ROS level, Fig. 3c). Lycopene (Sigma, St Louis, MO, USA) was dissolved in tetrahydrofuran (Sigma).

Reverse transcription–polymerase chain reaction (RT–PCR) and real-time RT–PCR analyses for IL-6 mRNA expression

Expression of IL-6 mRNA was assessed using RT–PCR analysis standardized by coamplification with the house-keeping gene GAPDH, which served as an internal control. Total RNA isolated from the cells was reverse transcribed into cDNA and used for PCR with primers specific for rat IL-6 and GAPDH. The IL-6 primer sequences were 5'-CTGGTCTTCTGGAGTTCCGTTTC-3' (forward primer) and 5'-CATAGCACACTAGGTTTGCCGAG-3' (reverse primer), giving a 302-bp PCR product. For GAPDH, the forward primer was 5'-ACCACAGTCCATGCCATCAC-3' and the reverse primer was 5'-TCCACCACCCTGTTGCTGTA-3', giving a 460-bp PCR product. After amplification for 28–30 cycles, the PCR products were separated on 1.5% agarose gel and visualized by UV transillumination.

IL-6 gene expression was quantified by real-time PCR using the Chromo4 detection system (MJ Research). cDNA was added to a SYBR Green Realtime PCR Master Mix (TOYOBO CO., Osaka, Japan) containing 30 pg/ml of IL-6 forward and reverse primers. The cDNA was amplified by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 45 s. During the first cycle, the 95°C step was extended to 1 min, and on the final cycle, the 65°C step was extended to 0.2 s to check the melting curve. The GAPDH gene was amplified in the same reaction to serve as the reference gene.

Electrophoretic mobility shift assay (EMSA) for NF- κ B activation

Cells were harvested, washed with PBS, and extracted with lysis buffer containing 10 mmol/l HEPES, 10 mmol/l KCl, 0.1 mmol/l ethylene diamine tetra acetic acid (EDTA), 1.5 mmol/l MgCl₂, 0.2% NP-40, 1 mmol/l dithiothreitol (DTT), and 0.5 mmol/l phenylmethyl sulfonyl fluoride (PMSF). The nuclear pellets were resuspended in nuclear extraction buffer containing 20 mmol/l HEPES, 420 mmol/l NaCl, 0.1 mmol/l EDTA, 1.5 mmol/l MgCl₂, 25% glycerol, 1 mmol/l DTT, and 0.5 mmol/l PMSF. The protein concentration of nuclear extract was determined by

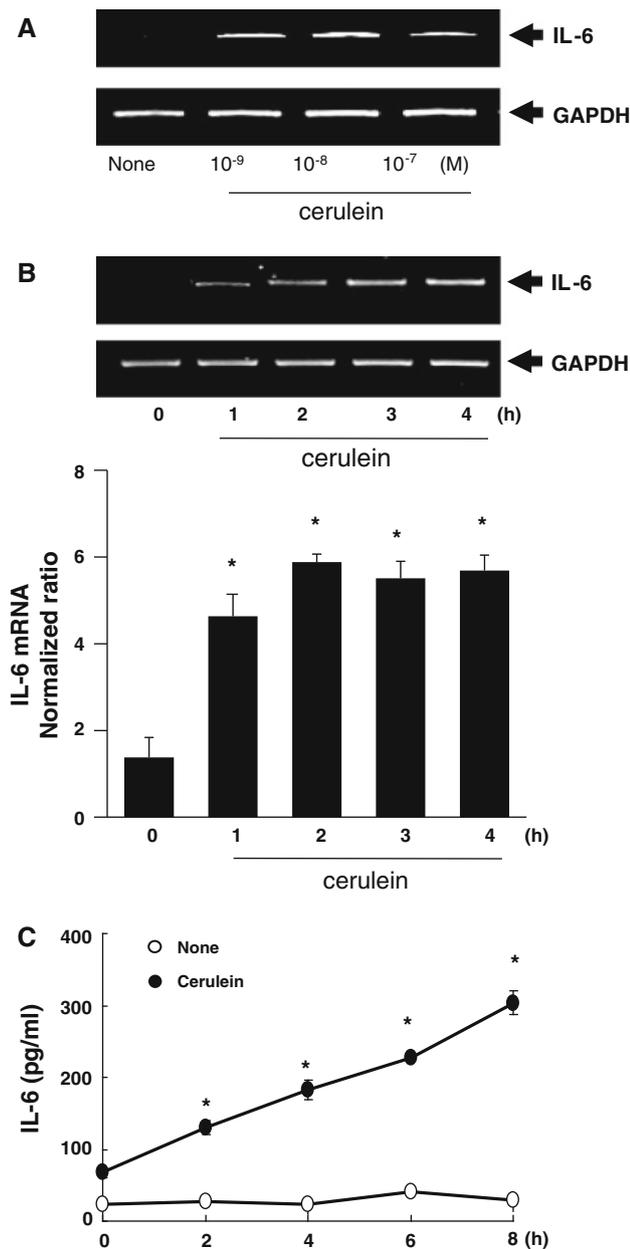


Fig. 1 Time-dependent expression of IL-6 in cerulein-stimulated pancreatic acinar cells. Acinar cells were treated with various concentrations of cerulein (10^{-9} – 10^{-7} M) for 3 h (**a**, mRNA expression by RT-PCR analysis). The cells were then stimulated with cerulein (10^{-8} M) for the indicated time periods for mRNA expression analysis by RT-PCR (**b**, upper panel) and real-time RT-PCR analysis (**b**, lower panel). * $P < 0.05$ versus 0 h. **c** The amount of IL-6 released into the medium from cerulein-stimulated pancreatic acinar cells was determined by ELISA. The values are expressed as means \pm S.E. of four different experiments. * $P < 0.05$ versus 0 h

the Bradford assay using Bio-Rad protein assay solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A NF- κ B gel shift oligonucleotide (Promega Corp, Madison, WI, USA) was labeled with [32 P] dATP using T4 polynucleotide kinase (GIBCO, Grand Island, NY, USA). The end-labeled

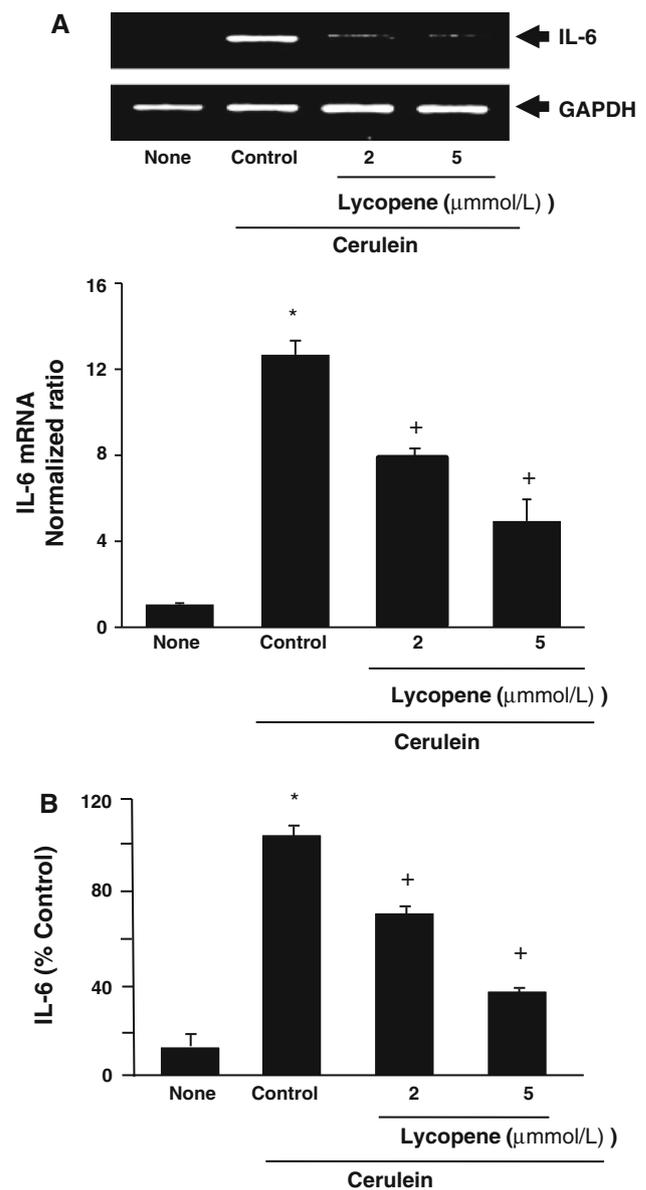


Fig. 2 Inhibitory effect of lycopene on IL-6 expression in cerulein-stimulated pancreatic acinar cells. Acinar cells were treated with lycopene (2 or 5 μ mol/l) for 2 h prior to stimulation with cerulein (10^{-8} M). The cells were stimulated with cerulein for 3 h (for mRNA expression, **a**) or 8 h (for IL-6 levels in the medium, **b**). mRNA expression of IL-6 was determined by RT-PCR analysis (**a**, upper panel) and by real-time RT-PCR analysis (**a**, lower panel). The IL-6 concentration in the medium was expressed as % of control. None, without cerulein stimulation; Control, cells stimulated with cerulein. * $P < 0.05$ versus none; + $P < 0.05$ versus control

probe was purified from unincorporated [32 P] dATP using a Bio-Rad purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer (TE). Nuclear extracts (2 μ g) were incubated with buffer containing 32 P-labeled NF- κ B consensus oligonucleotide for 30 min and subjected to electrophoretic separation on a nondenaturing acrylamide

gel. The gels were dried at 80°C for 2 h and exposed to a radiography film at −80°C.

Enzyme-linked immunosorbent assay (ELISA) for IL-6

The concentration of IL-6 in the medium was determined using enzyme-linked immunosorbent assay kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measurement of intracellular levels of ROS

Cells in a chamber slide (Nalge Nunc, Naperville, IL, USA) were pre-treated with lycopene for 2 h and stimulated with cerulein for 15 min. The cells were washed with HBS and then loaded with 5 µg/ml 2',7'-dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA) for 5 min. The fluorescent dichlorofluorescein was detected using a laser scanning confocal microscope (Leica TCS-NT, Heidelberg, Germany) with excitation and emission wavelengths of 488 and 520 nm, respectively [23]. The amount of ROS trapped in the cells was expressed relative to that of cells without cerulein stimulation, which was considered to be 1.

Statistical analysis

Statistical differences were determined using one-way ANOVA and Newman Keul's test. All values were expressed as means ± S.E. of four different experiments. A value of $P < 0.05$ was considered statistically significant.

Results

Cerulein induced IL-6 expression in pancreatic acinar cells in a dose-dependent manner

mRNA expression of IL-6 in cerulein-treated pancreatic acinar cells was determined using RT-PCR and real-time RT-PCR analyses. The cells were stimulated with various concentrations of cerulein (10^{-9} – 10^{-7} M) for 3 h (Fig. 1a). Cerulein induced expression of IL-6 mRNA at concentrations from 10^{-9} – 10^{-7} M, and maximum stimulation of IL-6 mRNA expression was observed in the cells stimulated with 10^{-8} M cerulein. When cells were treated with cerulein (10^{-8} M) for increasing time periods, IL-6 mRNA expression was evident from 1 h and increased up to 4 h by RT-PCR analysis (Fig. 1b, upper panel) and by real-time RT-PCR analysis (Fig. 1b, lower panel). GAPDH was constitutively expressed in pancreatic acinar cells and its expression was not changed by cerulein stimulation. As shown in Fig. 1c, the release of IL-6 into the medium

by cerulein-stimulated pancreatic acinar cells increased with time up to 8 h.

Lycopene inhibited IL-6 expression in cerulein-stimulated pancreatic acinar cells

Cells were treated with lycopene (2 or 5 µmol/l) for 2 h prior to cerulein stimulation for 3 h (for mRNA expression, Fig. 2a) or 6 h (for IL-6 levels in the medium, Fig. 2b). Cerulein-induced mRNA expression of IL-6 was inhibited by lycopene, as determined by RT-PCR analysis (Fig. 2a, upper panel) and by real-time RT-PCR analysis (Fig. 2a, lower panel). Lycopene also inhibited the cerulein-induced increase in IL-6 in the medium in a dose-dependent manner (Fig. 2b).

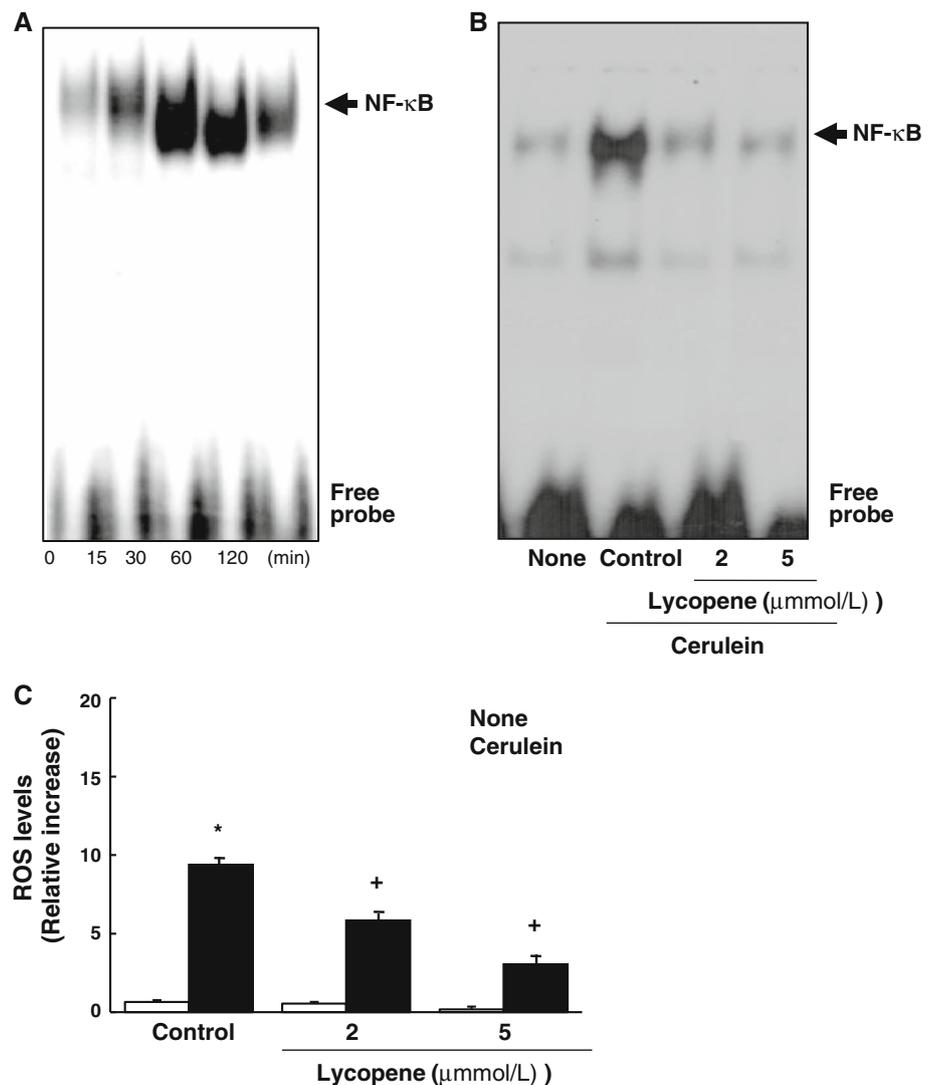
Cerulein-induced activation of NF-κB and increased ROS levels in pancreatic acinar cells was inhibited by lycopene

To determine whether lycopene suppressed activation of the oxidant-sensitive transcription factor NF-κB, we measured NF-κB-DNA-binding activity in nuclear extracts of pancreatic acinar cells. A time course for activation of NF-κB showed that cerulein induced NF-κB activation from 30 to 120 min (Fig. 3a). Lycopene inhibited the cerulein-induced activation of NF-κB at 30 min (Fig. 3b). Cerulein also induced an increase in intracellular levels of ROS (Fig. 3c), which was suppressed by treatment with lycopene in a dose-dependent manner.

Discussion

In the present study, we showed that lycopene has an inhibitory effect on NF-κB activation and IL-6 expression in pancreatic acinar cells, which is associated with inhibition of the increase in ROS levels in cells stimulated with cerulein. In previous studies on the antioxidant effect of lycopene, lycopene inhibited nitration of proteins and DNA strand breakage caused by peroxynitrite treatment [24] and decreased the oxidative DNA damage caused by the redox-cycling of catechol-estrogens [25] in Chinese hamster lung fibroblasts. In Hep3B cells treated with H₂O₂, lycopene suppressed DNA damage in a dose-dependent manner [26]. Liu et al. [27] determined the subcellular localization of lycopene in prostate cancer cells and found that 81% of the lycopene was localized to the nucleus. These results support an antioxidant effect of lycopene in various cells. The present study indicates that the inhibitory effect of lycopene on cerulein-induced activation of NF-κB may be caused by the ROS scavenging activity of lycopene in the cells.

Fig. 3 Inhibitory effect of lycopene on NF- κ B activation and ROS production in cerulein-stimulated pancreatic acinar cells. Acinar cells were stimulated with cerulein (10^{-8} M) for the indicated time periods (a). The cells were treated with lycopene (2 or 5 μ mol/l) for 2 h prior to stimulation with cerulein (10^{-8} M) for 30 min for determination of NF- κ B activation by EMSA (b) or 15 min for measurement of intracellular levels of ROS (c). DCF fluorescence for ROS was detected on a laser scanning confocal microscope. The amount of ROS trapped in the cells was expressed as the increase relative to that of cells without cerulein stimulation, which was considered to be 1 (None). The values are expressed as means \pm S.E. of four different experiments. * $P < 0.05$ versus none control (without lycopene and without cerulein stimulation). + $P < 0.05$ versus cerulein control (without lycopene and with cerulein stimulation)



The first reported *in vivo* biological activities of lycopene were protection of mice against bacterial infection [28] and protection against radiation and development of specific types of ascites tumors [29]. Recent studies have focused more on the beneficial effect of lycopene in prostate cancer chemoprevention [30]. Lycopene supplementation has been shown to reduce IL-6 levels in disabled older women [31]. In addition, lycopene interferes with autocrine/paracrine loops in the Dunning prostate cancer model [32] and inhibits the expression of IL-6 in prostate cancer [32, 33].

Lycopene inhibits lipopolysaccharide-induced activation of NF- κ B by suppressing the phosphorylation of I- κ B and thus protecting it from degradation. The inhibitory effect of lycopene on NF- κ B activation results in inhibition of IL-6 expression in prostate cancer [34]. Lycopene shows inhibitory effects on TNF- α -induced activation of NF- κ B and ICAM-1 expression in human umbilical endothelial

[35]. Moreover, lycopene inhibits the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells by suppressing the activation of NF- κ B and mitogen-activated protein kinases [36]. These studies demonstrate a possible relationship between NF- κ B activation and IL-6 expression, indicating that inhibition of NF- κ B activation may result in inhibition of IL-6 expression.

Regarding the anti-inflammatory mechanisms of lycopene, Palozza et al. have recently shown that lycopene increases levels of peroxisome proliferator-activated receptor γ (PPAR γ) concomitant with inhibition of ROS production and NF- κ B activation in a model of atherosclerosis [37]. The PPAR γ receptor agonist rosiglitazone has been found to alleviate the inflammatory response in experimental-induced pancreatitis [38]. Moreover, in a cerulein-treated pancreatic acinar cell model, the PPAR γ receptor agonist pioglitazone attenuates NF- κ B activity [39].

Since NF- κ B activation is induced by oxidants, the antioxidant effect of lycopene may contribute to inhibition of NF- κ B activation and IL-6 expression in pancreatic acinar cells. Lycopene may be beneficial in the prevention and/or treatment of acute pancreatitis by inhibiting activation of NF- κ B and the expression of inflammatory cytokines in pancreatic acinar cells. Further studies should be performed to investigate the precise mechanism by which the potent antioxidant lycopene exerts anti-inflammatory effects in pancreatic acinar cells by determining the oxidant-mediated inflammatory signaling pathways involved.

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