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THE EFFECT OF LIPOPOLYSACCHARIDE ON ENHANCED INFLAMMATORY PROCESS WITH AGE: MODULATION OF NF-κB

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ABSTRACT

Oxidative stress is thought to be a causative factor for age-related damage in a wide variety of cellular constituents that can lead to dysfunction and various pathological conditions, including the inflammatory process. At the molecular level, the redox-sensitive transcription factor, NF-κB plays a key role in the regulation of the inflammatory process, along with cytokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). We studied the mechanism underlying the modulation of the inflammatory reaction with age by investigating NF-κB activation and the expression of COX-2, iNOS, and cytokines genes in hepatic tissues isolated from young and old rats. We expanded our investigation of these factors in rats injected with the inflammatory activator, lipopolysaccharide (LPS). Data showed that NF-κB activity was up-regulated with age and was further enhanced by LPS injection, indicating an increased susceptibility and sensitivity to the inflammatory stimulus with age. To explore further the molecular events leading to NF-κB activation, we investigated the inhibitory component of NF-κB complex, IκB. Cytosolic IκBα, but not IκBβ, was significantly decreased in both old and LPS-treated rats, signifying the enhanced migration of cytosolic NF-κB complex into the nucleus following dissociation from the inhibitor. The appearance of the polypeptide, p65, as determined in the nucleus, corresponded with the change in IκBα, providing further supporting evidence for the molecular process involved in NF-κB activation. Our additional investigation of two proinflammatory-related enzymes, COX-2 and iNOS, and three cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α, clearly showed aged-related increases, in corroboration with the NF-κB activation. Our results demonstrated that LPS injection caused the enhanced gene expression of inducible proinflammatory proteins, COX-2 and iNOS through NF-κB activation.

INTRODUCTION

Aging processes are characteristically described as time-dependent functional declines that lead to the cell's incapacity to withstand external and internal changes. Among the modern hypotheses for aging phenomena, the free radical based oxidative stress hypothesis of aging postulated an imbalance in the net result of oxidative stress and counteracting anti-oxidative forces to be responsible for the characteristic changes of the aging process (1, 2). Age-related increase in oxidative stress can cause oxidative damage to cellular structure and tissue damage, leading to a disturbance in cellular homeostasis (3).

Recent reports show oxidative processes to be involved in the inflammatory process associated with aging and age-related diseases (4). Tissue damage induced by increased reactive oxygen species (ROS) is likely to be accompanied by inflammatory reactions. Their activation accompanies the reintroduction of neutrophils, which release lytic enzymes that directly induce tissue damage and proinflammatory mediators that amplify the local inflammatory reaction (5). As a part of inflammatory process, oxidizing agents such as ROS produced by phagocytic-invading leukocytes causes the exacerbation of tissue damage. One of the major

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physiological changes that take place as a consequence of aging is an impaired immune surveillance system to optimally monitor environmental stresses (6, 7). This decline in immune function has been directly and indirectly linked to increased susceptibility to infections, coupled with imbalanced inflammatory reactivities (8). Age-related changes in levels of cytokines investigated in *in vitro* systems are controversial (9-11). The production of IL-6, but not IL-1β and TNFα in peripheral blood mononuclear cells, was reported to be increased in elderly compared to young subjects (9), although Riancho *et al.* (10) reported an age-related increase of IL-1 production in peripheral blood mononuclear cells. IL-1β levels were reported higher and IL-6 levels lower in old rats compared to young rats (11). It has also been observed that an age-associated increase in cyclooxygenase (COX) products in macrophages may be due to increased COX-2 expression (12). Although iNOS has been shown to be up-regulated in old rat brain (13) and blood vessels (14), the effect of aging on liver iNOS levels has been not investigated in the liver.

In a previous study, our laboratory reported that NF-κB, COX, and inducible NO synthase (iNOS) were up-regulated in the kidney during the aging process (15, 16) and that this up-regulation correlated with increase in age-related oxidative status. Based on these and other data, we recently proposed the "Inflammation Hypothesis of Aging" with the hope of providing a new perspective into the molecular insights that bridge aging and age-related diseases (17).

Under the influence of the inflammatory response, several major inflammatory enzymes including COX-2 and iNOS are activated. COX, the enzyme catalyzing oxidation of arachidonic acid, exists in two isoforms, COX-1 and COX-2. COX-1 is generally considered a constitutive enzyme involved in prostagladins (PGs) synthesis for the regulation of physiological functions (18). The inducible COX-2, responsive to proinflammatory stimuli such as lipopolysaccharide (LPS), several cytokines, growth factors, and tumor promoters (19-21) plays a key role in the production of proinflammatory PGs (20).

The redox-sensitive and oxidant stress-activated transcription factor, NF-κB is an important modulator of inflammation that transcriptionally controls the production of cytokines, adhesion molecules, immunoreceptors, and acute phase proteins (22). In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50kDa (p50) and 65kDa (p65), which are non-covalently associated with cytoplasmic inhibitory proteins, including IκBα and IκBβ. Following translocation into nucleus, NF-κB up-regulates the transcription of various genes involved in the immune and inflammatory responses. For example, NF-κB regulates the gene expression of proinflammatory cytokines, such as IL-1β (23), IL-6 (24), and TNFα (25), and inflammatory enzymes, including COX-2 (26) and iNOS (27), through κB sites in their promoter region. Since these inflammatory reactions can be induced by lipopolysccharide (LPS) and cytokines, it is of interest to investigate the LPS-induced inflammatory phenomena in relation to aging.

The present study was carried out to systemically access at first levels of proinflammatory proteins such as IL-1β, IL-6, TNFα, COX-2 and iNOS, and then investigate the molecular mechanisms underlying the increased expression of these proteins in the aged and LPS-challenged rats. The study further probed attempted to establish that the aging process induces IκBα proteolysis and facilitates nuclear p65 translocation through the modification of NF-κB.

RESULTS

ROS generation and LPS-treated rat liver

To investigate the effect of age and LPS induced-inflammation on oxidative stress, the ROS generation was measured. Total ROS generation was assayed by measuring the oxidation of DCFDA to DCF by ROS. As shown in Fig. 1, fluorescence intensities in young rats (CY), old rats (CO), LPS-treated young rats (LY), and LPS-treated old rats (LO) groups were 0.329±0.027, 0.440±0.049, 0.415±0.041, and 0.503±0.092 /min/mg protein, respectively. Results show that more ROS was generated increased in old rats compared to young rats. LPS caused further increased ROS generation in old rats, aggravating the age-related oxidative status.

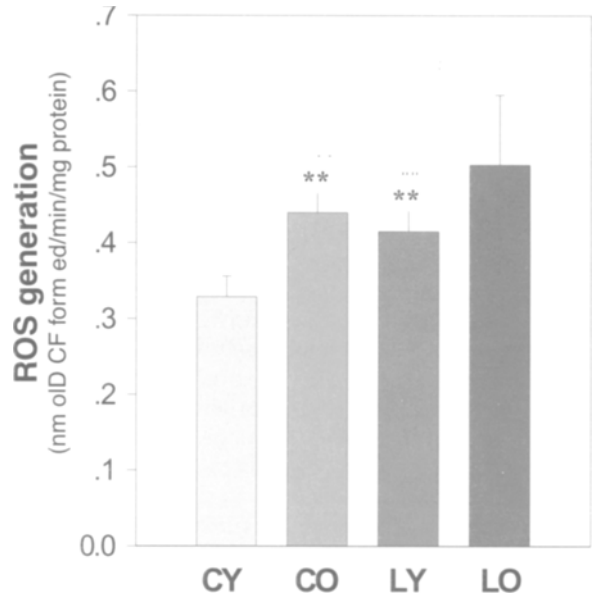

Figure 1: Effects of age and LPS on ROS generation. Each value is the mean ± S.E. of 5 rats. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats. Statistical significance: **p<0.01 *vs.* young rats.

Cytokine gene expression during aging and inflammatory processes

Proinflammatory cytokines such as IL-1β, IL-6, and TNFα are key promoters of immunologic and inflammatory responses. To investigate changes in the gene expression of these cytokines with age and LPS, we measured mRNA levels by RT-PCR (Fig. 2-a). The mRNA levels of IL-1β, IL-6, and TNFα showed increases in old rats and

was further enhanced by LPS treatment. The protein levels of these cytokines were similar to the mRNA levels (Fig. 2-b).

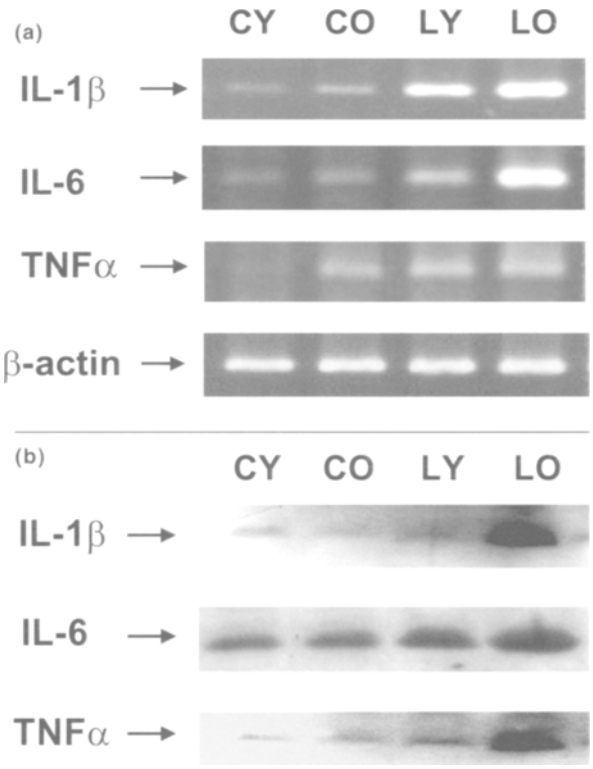

Figure 2: Effects of age and LPS on the protein levels (a) and mRNA levels (b) of IL-1β, IL-6, and TNFα. **(a)** Cytosolic fractions of each group were resolved in a 15% SDS-polyacrylamide gel electrophoresis and Western blot was performed using antibodies of IL-1β, IL-6, and TNFα. Bands were visualized using ECL procedure. **(b)** RT-PCR was performed to determine levels of mRNA of IL-1β, IL-6, and TNFα. β-Actin was used for positive control. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats.

Gene expression during aging and inflammatory processes.

Fig. 3-a and b show the gene expression levels of inflammatory enzymes, indicating iNOS and COX-2. COX-2 is slightly increased in old and LPS-treated young rats, but show the highest expression in LPS-treated old rats. However, COX-1 level was not influenced. The protein levels of COX-1 and COX-2 were similar to those seen at their mRNA levels. As shown in Fig. 4-a and b, both mRNA and iNOS protein levels that were expressed at a low level in young rats increased by both aging and LPS challenge. Constitutive cNOS expression was increased by LPS challenge, but not by aging, which is an interesting finding.

Activation of NF-κB by aging and LPS challenge

Since redox-sensitive NF-κB has been known to regulate the expression of proinflammatory proteins, we investigated the modulation of this transcription factor by LPS and age. EMSA showed that the NF-κB activity was up-regulated in old rats compared to young rats. In

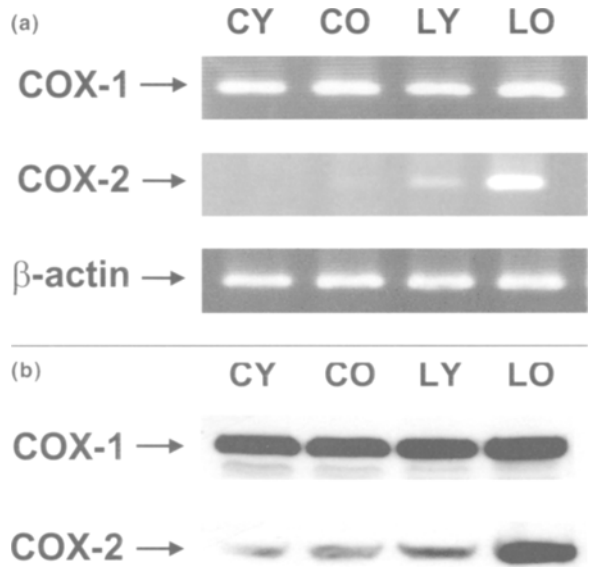

Figure 3: Effects of age and LPS on protein levels (a) and the mRNA levels (b) of COX-1 and COX-2. **(a)** Postmitochondrial fractions of each group were resolved in a 10% SDS-polyacrylamide gel electrophoresis and Western blot was performed using antibodies of COX-1 and COX-2. Bands were visualized using ECL procedure. **(b)** RT-PCR was performed to determine levels of mRNA of COX-1 and COX-2. β-Actin was used for positive control. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats; COX.

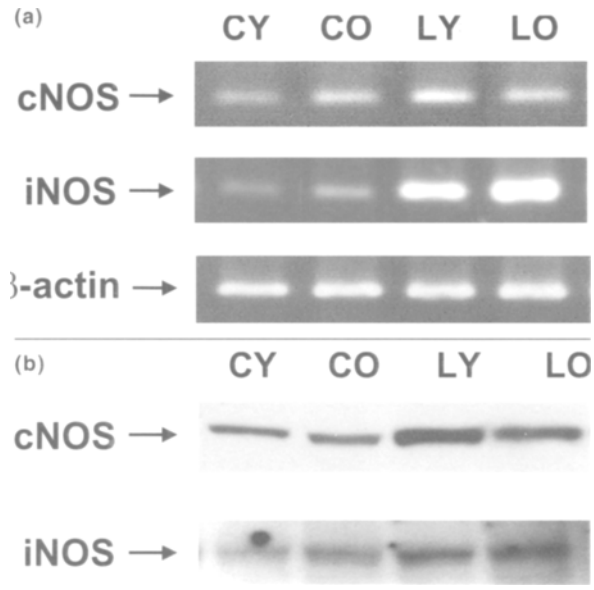

Figure 4: Effects of age and LPS on the protein levels (a) and mRNA levels (b) of cNOS and iNOS. (a) Postmitochondrial fractions of each group were resolved in a 6% SDS-polyacrylamide gel electrophoresis and Western blot was performed using antibodies of cNOS and iNOS. Bands were visualized using ECL procedure. (b) RT-PCR was performed to determine levels of mRNA of COX-1 and COX-2. β-Actin was used for positive control. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase.

addition, this age-related activation was enhanced by LPS challenge, indicating the organism's increased

sensitivity to proinflammatory stimuli with age (Fig. 5-a). To ascertain the NF-κB binding specificity, supershift and competition analysis were performed (Fig. 5-b). Supershifting experiments performed with antibodies directed toward NF-κB proteins demonstrated that NF-êB is formed mainly of p50 and p65 subunits. The treatment with p50 antibody resulted in a supershift with the observed protein-DNA complex, and the upper band disappeared by treatment with p65 antibody. Competition analysis with excess, unlabeled oligonucleotide showed that the binding is specific (Lane 5 in Fig . 5-b).

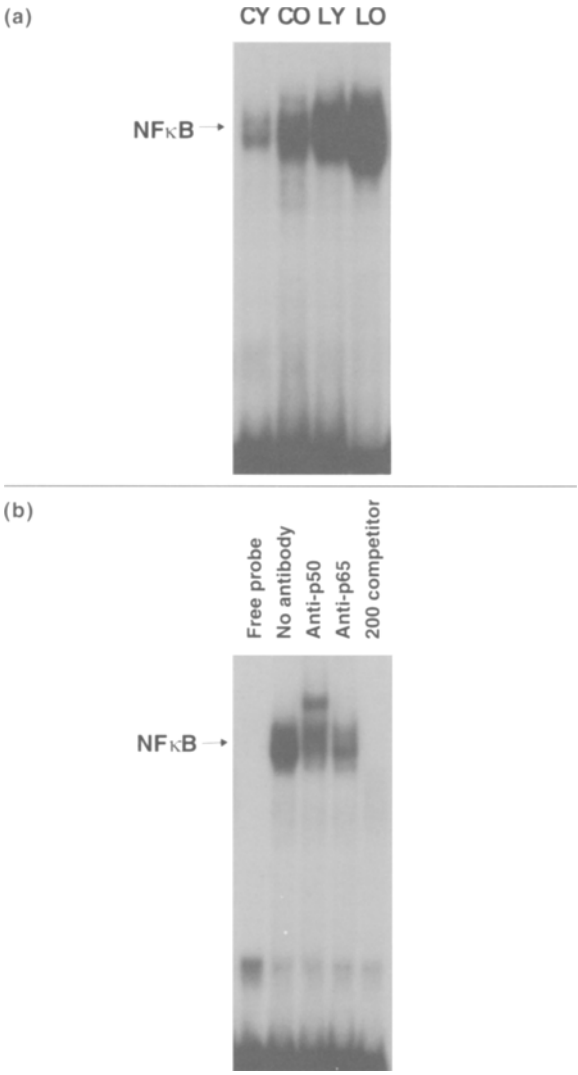

Figure 5: Effects of age and LPS on the NF-κB DNA binding activity (a) and supershift/competition analysis to determine specificity of NF-κB binging (b). Nuclear extracts of each group were incubated with ^{32}P-end labeled probes containing a binding site for NF-κB. Resulting complexes were separated by electrophoretic mobility shift assay. Nuclear extracts were incubated with the antibodies raised against p50 and p65 or excess unlabeled NF-κB oligonucletides (200-fold competitor) for 20 min before the addition of labeled probe, and then, electrophoretic mobility shift assay was performed. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats.

Changes in protein components of NF-κB with aging and LPS challenge.

To delineate underlying the potential pathways for NF-κB activation, we investigated degradation of the IκB proteins modulated by aging and LPS challenge. Because IκBα and IκBα are mainly expressed in the liver (28), we carried out Western blot analysis on IκBα and IκBβ with cytoplasmic extracts. Results in Fig. 6-a show that the protein level of IκBα decreased in aged and LPS-treated tissues in parallel to the NF-κB activity. However, IκBβ protein levels showed no noticeable differences except for a modest decrease in the LPS-treated groups. These data were taken as a strong indication that aging- and LPS-induced NF-κB activation is induced by IκBα degradation.

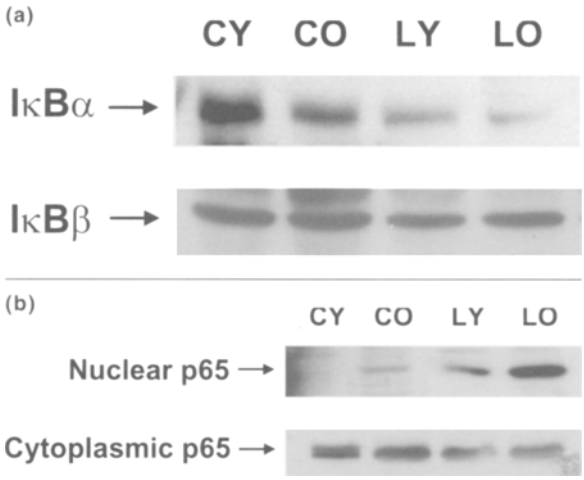

Figure 6: Effects of age and LPS on the protein levels of I B and I B . (a) Cytosolic fractions of each group were resolved in a 12% SDS-polyacrylamide gel electrophoresis and Western blot was performed using antibodies of IκBα and IκBβ. (b) Nuclear and cytosolic fractions of each groups were resolved in a 10% SDS-polyacrylamide gel electrophoresis and Western blot was performed using p65 antibody. Bands were visualized using ECL procedure. CY, young rats; CO, old rats; LY, LPS-treated young rats; LO, LPS-treated old rats.

To establish whether the activated proteolysis of IκBα resulted in an increase of the nuclear translocation of NF-κB complex, nuclear p65 protein levels were examined by Western blot analysis. Results shown in Fig. 6-b indicate that the amount of p65 in nuclei was increased by aging and LPS, corresponding with NF-κB activity and the cytoplasmic IκBα protein level. The cytosolic p65 disappearance, coupled with the increase of nuclear p65, signifies translocation of p65 from cytoplasm to nucleus.

DISCUSSION

Research has shown that ROS are important mediators involved in the inflammatory process. Many ROS such as $O_2^{\cdot}$, H_2O_2, and •OH are produced by activated macrophages and neutrophils under normal conditions in the host as a defense against invading microorganisms (29). It becomes increasingly apparent that ROS are closely involved in the initiation and/or amplification of inflammatory reactivity via the up-regulation of various genes for proinflammatory cytokines, adhesion molecules, or inflammatory enzymes. It is important noticing that the activation of several transcription factors

such as NF-κB is required for the gene expression of an inflammatory mediator (4). Based on new revelations, we recently have proposed "Inflammation Hypothesis of Aging" (4, 17). Data generated from the present study re-enforce the hypothesis by providing stronger evidences for the involvement of proinflammatory reactions in the aging process.

These biological effects of LPS are mediated by the immune system and in particular by the cells of the monocytic lineage (30, 31). LPS activates monocytes/macrophages and other cells to induce the expression of proinflammatory cytokines such as IL-1β, IL-6, and TNFα via NF-κB activation (32-35). The signal transduction pathway initiated by LPS binding with its receptor to NF-κB activation is closely related to ROS generation and oxidative status, as demonstrated by inhibitory effects of antioxidants such as N-acetylcysteine (NAC) and pyrrrolidine dithiocarbamate (PDTC) upon NF-κB activation (35).

In our study, with age, LPS-induced changes increase ROS generation is consistent with other work (3, 36, 37), showing increased oxidative stress in the old rat (Figure 1).

The molecular exploration of proinflammatory proteins upon activation by ROS revealed that ROS-sensitive, NF-κB loci are part of the binding process to the promoters of the encoding genes (23-27, 38). A result of EMSA on NF-κB showed that the NF-κB binding activity was up-regulated to a greater degree in old rats compared to young rats. Since the activation of the NF-κB complex depends on the dissociation of the inhibitor protein, IκB from the complex, it is important to access the cytoplasmic IκB status. As shown in Fig. 6-a, the amount of IκBα was significantly lower in old and/or LPS-treated rats, while the change in the IκBβ level was minimal indicating IκBα's a bigger role than IκBβ in the regulation of NF-κB activity in aging and LPS-challenged rats.

The increased degradation of IκBβ allows the translocation of NF-κB complex to the nucleus. The nuclear p65 amounts were shown to increase by aging and LPS, which corresponded with the levels of NF-κB activity and cytoplasmic IκBα protein levels observed. The cytosolic p65 decreased in a parallel manner with the increase of nuclear p65, demonstrating translocation of p65 from cytoplasm to the nucleus. These findings indicate that the increase in the DNA-binding activity of NF-κB by aging and LPS is likely responsible for the increase of NF-κB translocation to the nucleus via increased IκBα degradation. In addition, we have shown that the activity of 26S proteasome, reportedly responsible for the degradation of the ubiquitinated IκB, decreases with age in rat livers (39). Other investigators have reported that total proteasome activity declines with age (40, 41). Thus, it seems that a rate-limiting factor for the apparent increase in the degradation of IκBα in aged animals is not the proteasome. It is possible that a kinase in a series of phosphorylation steps makes IκB susceptible to ubiquitination and/or ubiquitination itself is responsible.

The major significances of our findings are LPS challenge exacerbates the inflammatory process of the aged rats through the induction of oxidative stress, responsive genes including IL-1β, IL-6, TNFα, COX-2 and iNOS. And our finding showing the enhanced inflammatory reaction is mediated through the activation of NF-κB is of importance. Furthermore, our study also delineated the modulation of NF-κB action by increased IκBα degradation that likely plays a key role in the inflammatory process underlying aging.

In conclusion, we described the effect of LPS on the role of NF-κB in the regulation of inducible proinflammatory proteins during the aging process. Understanding the regulatory mechanisms of the proinflammatory proteins during aging should provide molecular insights for the inflammatory process in many age-related disease including atheroscleosis, diabetes, vascular dysfunctions and Alzheimer's.

EXPERIMENTAL PROCEDURES

Animals and LPS treatment procedure

Specific pathogen-free (SPF) male Fischer 344 rats at 13 and 31 months of age were separated into young (6 rats) and old (6 rats) groups, respectively. To investigate the effects of inflammation on the aging process, two additional groups were used for the injection of bacterial lipopolysaccharide (LPS). LPS was injected intraperitonially with 5 mg/kg body weight doses in young and old rats. After 5 hrs, rats were sacrificed by decapitation and the livers quickly removed. The tissue was immediately immersed in liquid nitrogen and stored at -80°C.

Materials

Most chemical reagents used in the study were purchased from Sigma Chemical Company (St. Louis, MO, USA), except as noted. 2',7'-Dichlorofluorescein diacetate (DCFDA) was from Molecular Probes, Inc. (Eugene, OR, USA). RNAzol™B was obtained from TEL-TEST, INC. (Friendwood, TX, USA). Primers of IL-1β, IL-6, TNFα, and IκBα for RT-PCR were synthesized by BIOBASIC (Ontario, Canada). Primers of COX-1, COX-2, iNOS, cNOS, IκBα, and β-actin were synthesized by Bioneer (Daejeon, Korea). Anti-IL-1β, anti-IL-6, anti-TNFα, anti-COX-1, anti-COX-2, anti-iNOS, anti-p65, anti-IκBα, and anti-IκBβ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cNOS antibody was obtained from Transduction Lab. (Lexington, KY, USA). A horseradish peroxidase-conjugated donkey anti-rabbit antibody was obtained from Amersham (Bucks, UK) and a horseradish peroxide-conjugated donkey anti-goat antibody from Serotec (Oxford, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Bedford, MA, USA). The radionucleotide [γ-^{32}P]-ATP was obtained from Amersham (Bucks, UK).

Methods

A. Tissue preparation

One gram of liver was homogenized in 10 ml of homogenizing buffer (50 mM potassium phosphate

buffer containing 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM pepstatin, 80 mg/L trypsin inhibitor, pH 7.4) and centrifuged at 900 g at 4°C for 15 min. The supernatants were re-centrifuged at 12000 g at 4°C for 15 min. The pellets are referred to as mitochondrial fraction and supernatants are referred to as postmitochondrial fraction. The supernatants were re-centrifuged at 105,000 g at 4°C for 1 hr. These pellets are referred to as microsomal fraction and three supernatants as cytosolic fractions. All fractions were stored at -80°C.

B. Assay for ROS generation

ROS generation was measured as previously described (42). DCFDA (25 μM) was added to homogenate, mitochondrial fraction, and postmitochondrial fraction. Changes in fluorescence were measured at excitation wavelength of 485 nm and an emission wavelength of 530 nm by Fluorescence Plate Reader (BIO-TEK Instruments, Inc., Winooski, USA) for 30 min.

C. Reverse transcriptase-polymerase chain reaction assay (RT-PCR)

1) Isolation of RNA

Tissue samples were homogenized in the presence of RNAzol™B (2 ml per 100 mg tissue) with a few strokes in a polytron homogenizer. Aliquots of 0.2 ml chloroform per 2 ml homogenate was added, the samples were covered tightly, shaken vigorously for 15 sec and stayed on ice for 5 min, then centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to the fresh tube and an equal volume of isopropanol was added. After shaking, the mixture was let stand for 15 min at 4°C. Following centrifuging at 12,000 g at 4°C for 15 min, the supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequently centrifuged at 7,500 g at 4°C for 8 min. The pellet was dried for 10-15 min. The RNA pellet was dissolved in DEPC-treated water (43).

2) Reverse transcription procedure

The first strand cDNA was synthesized from 2 μg total RNA. DEPC-treated water and 250 ng random primer were added, incubated at 75°C for 5 min and then let stay on ice for 5 min. The mixture containing 10 mM DTT, 1X first strand buffer, 0.5 mM dNTP, 100 U reverse transcriptase, and 16.5 U of RNase inhibitor were added and incubated at 37°C for 2 hr. The reaction was stopped by boiling at 100°C for 2 min, and cDNA was stored at -20°C until use.

3) Procedure for polymerase chain reaction

To carry out the PCR, gene products amplification was performed in a PCR buffer (Promega, Madison, WI, USA) containing 200 μM dNTP, 0.25 U of Taq DNA polymerase, and 50 ng of sense primer and anti-sense primer per reaction. Amplification reactions were held at 95°C for 'hot-start' PCR for 5 min and run in an automated thermal cycler. The primer pairs used and the number of cycles for particular proteins are specified in Table 1. Each cycle consisted of denaturation at 4°C for 30 sec, annealing at 4°C for 30 sec, and extension 72°C for 1 min. Electrophoresis was performed in 1.5% agarose gel. After suitable staining with ethidium bromide solution, the gel was observed under U.V. transilluminator.

Table 1. Oligonucleotides sequences used and number of cycles for PCR amplification.

Gene		Sequence	Size (bp)	Cycles
IL-1β	ss	5' CCA GGA TFA GGA CCC AAG CA 3'	519	39
	as	5' TCC CGA CCA TTG CTG TTT CC 3'		
IL-6	ss	5' CTT CCA GCC AGT TGC CTT CT 3'	496	30
	as	5' GAG AGC ATT GGA AGT TGG GG 3'		
TNFα	ss	5' CGA GTG ACA AGC CCGTAG CC 3'	468	39
	as	5' GGA TGA ACA CGC CAG TCG CC 3'		
COX-1	ss	5' CTG CAT GTG GCT GTG GAT GTC ATC 3'	441	39
COX-2	ss	5' CAA GCA GTG GCA AAG GCC TCC ATT 3'	474	42
	as	5' TAG TCT GGA GTG GGA GGC ACT TGC 3'		
cNOS	ss	5' TTC CGG CTG CCA CCT GAT CCT AA 3'	614	30
	as	5' AAC ATG TGT CCT TGC TCG AGG CA 3'		
iNOS	ss	5' AAT TCA CAG CTC ATC CGG TAC 3'	678	29
	as	5' AGA TAG GAC ATA GTT CAA CAT 3'		
β-actin	ss	5' TGG AGA AGA TTT GGC ACC AC 3'	423	23
	as	5' AGT CTA GGG CAA CAT AGC AC 3'		

ss,sense; as, antisense

D. Western blotting procedure

Samples were boiled for 5 min with gel loading buffer (0.125 M Tris-Cl, 4% SDS, 10% 2-mercaptoethanol, pH 6.8, 0.2% bromophenol blue) in ratio of 1:1. Total protein-equivalents for each sample were separated on sodium dodecyl sulphate-polyacrylamide mini-gel using the Laemmli buffer system and transferred to PVDF membrane at 15 V for 1.5 hr in a wet transfer system. Nonspecific binding to the membrane was blocked by non-fat milk-blocking buffer in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20 at room temperature for 1 hr. The membrane was washed and then probed with rabbit or goat polyclonal antibodies against specific proteins overnight at 25°C. The secondary antibodies used were horseradish peroxidase-conjugated donkey anti-rabbit antibody and anti-goat antibody. Membranes were developed with enhanced chemiluminescence detection system and exposed to Hyperfilm. Prestained blue protein markers were used for molecular weight determinations (44).

E. Nuclear extract preparation

All solutions, tubes, and centrifuges were maintained at 0-4°C. The preparation of rat liver nuclear extracts was based on by Hattori *et al* methods' (45). The nuclear extract was frozen at 80°C in aliquots until electrophoretic mobility shift assay (EMSA) and Western blotting were done. The concentration of total protein in samples was measured with bicinchoninic acid.

F. Procedure for electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of NF-κB transcription factors in nuclear extracts (46). The oligonucleotide used to detect the DNA binding activities of NF-κB was as follows: 5-AGCTTCAGAGGGGATTTCCGAGAGG-354). Complementary oligonucleotides synthesized

separately were annealed in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 10 mM $MgCl_2$ after end-labeling with T4 polynucleotide kinase (Promega) and the radionucleotide [γ-^{32}P]-ATP (Amersham). Nuclear extracts (10 μg) were preincubated in a reaction mixture containing 5% glycerol, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 1.0% (v/v) Nonidet P40 and 1μg poly (dI-dC) on ice for 15 min and then incubated with 1.6 pmol (50,000 cpm) of ^{32}P-end-labeled oligonucleotide at room temperature for 20 min. The nuclear protein-^{32}P-labeled oligonucleotide complex was separated from free ^{32}P-labeled oligonucleotide by electrophoresis through a 6% native polyacrylamide gel in a running buffer of 0.5X TBE (50 mM Tris, pH 8.0, 45 mM borate, 0.5 mM EDTA). After separation, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film exposing at -80°C for 1-2 days.

7. Statistics

Results were analyzed statistically by one-way ANOVA analysis. Values of $p < 0.05$ were considered statistically significant.

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ABBREVIATIONS

NF-κB: Nuclear Factor-κB
COX-1: cyclooxygenase-1
COX-2: cyclooxygenase-2
iNOS: inducible nitric oxide synthase
LPS: lipopolysaccharide
IκB: inhibitor κB
IL-1β: interleukin-1β
IL-6: interleukin-6
TNFα: tumor necrosis factor- α
ROS: reactive oxygen species
PGs: prostaglandins
DCFDA: 2',7'-dichlorodihydrofluorescein diacetate
DCF: 2',7'-dichlorodihydrofluorescein
NAC: N-acetylcysteine
PDTC: pyrrolidine dithiocarbamate
SPF: specific pathogen-free
DEPC: diethylpyrocarbonate

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