ELSEVIER

Journal of Nutritional Biochemistry 15 (2004) 210–219

THE JOURNAL OF Nutritional Biochemistry

Suppression of steroidogenesis and activator protein-1 transcription factor activity in rat adrenals by vitamin E deficiency–induced chronic oxidative stress

Parveen Abidi[a], Susan Leers-Sucheta[a], Salman Azhar[a,b,*]

[a]*Geriatric Research, Education and Clinical Center (GRECC), Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA*
[b]*Department of Medicine, Division of Gastroenterology and Hepatology, Stanford University School of Medicine, Stanford, CA 94305, USA*

Received 5 July 2003; received in revised form 22 October 2003; accepted 13 November 2003

Abstract

Excessive oxidative stress and associated macromolecular damage are considered to be key features of aging, and appear to contribute to the age-related decline in steroid hormone production in adrenal and testicular Leydig cells. The current studies were initiated to examine the potential mechanism by which excessive oxidative stress during aging attenuates the functional expression of the oxidant-responsive transcription factor Activator protein-1. Chronic oxidative stress was induced in vivo by maintaining groups of rats on a diet deficient in vitamin E for 6 months. Plasma, liver, and adrenal tissues from vitamin E–deficient animals had negligible levels of this vitamin and showed high susceptibility to in vitro lipid peroxidation. Synthesis and secretion of corticosterone in response to corticotropin (ACTH), dibutyryl-cAMP, or 20α-hydroxycholesterol in vitro was significantly reduced in adrenocortical cells prepared cells from rats deficient in vitamin E. AP-1 DNA-binding activity was diminished ~55 % in adrenal extracts from vitamin E–deficient rats with no corresponding change in the binding activity of SP-1. The vitamin E deficiency–mediated loss of AP-1 activity was not due to an alteration in the dimeric composition of constituent proteins, but rather to a general down-regulation of steady-state levels of members of the Fos and Jun families of proteins. Interestingly, vitamin E deficiency also reduced the expression of the redox-regulated Ref-1 protein. Collectively these data demonstrate that chronic oxidative stress specifically down-regulates essential components of the AP-1 transcription factor complex, and suggest that aberrancies in AP-1 expression may adversely affect processes crucial for intracellular cholesterol transport and steroid hormone production.

Keywords: Corticosterone; Lipid peroxidation; APE/Ref-1; Fos/Jun family proteins; α-Tocopherol; Cholesterol

1. Introduction

Abundant evidence indicates that advancing age in humans [1–3] and experimental animals [4–6] is associated with a profound change in the synthesis and secretion of steroid hormones. Previous work from this laboratory has suggested that the major cause for the age-related decline in steroid hormone production in adrenal or testis is the inability of aging organs to efficiently mobilize and transport intracellular cholesterol to mitochondria, as is required for steroid hormone production [7–12]. However, various cellular and molecular mechanisms controlling this defect have not been definitely identified.

Reactive oxygen species (ROS) have long been implicated in the aging process [13–16]. The potential risk for damage to macromolecules [17–19] from excessive oxidative stress is especially high for steroidogenic cells, which use molecular oxygen not only for aerobic energy production (ATP synthesis) in mitochondria but also for steroid biosynthesis, and thus exhibit high rates of oxidative metabolism and generation of ROS such as oxygen-centered free radicals and peroxides, which are by-products of metabolism [4,20–22]. Because lipid peroxidation–mediated oxidative damage of cellular membranes can affect membrane structure and/or fluidity, and because virtually every event associated with cholesterol processing and steroidogenesis is dependent on the integrity of cell membranes [23–25], the likelihood of steroidogenesis being adversely affected is quite high. As a result, steroidogenic organs are especially well equipped with both nonenzynmatic (e.g.,

* Corresponding author. Tel.: (650) 858-3988; Fax: (650) 496-2505.
E-mail address: salman.azhar@med.va.gov (S. Azhar).

0955-2863/04/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2003.11.007

vitamin C, vitamin E, reduced glutathione) and enzymatic (e.g., superoxide dismutases, catalase and glutathione peroxidase) antioxidant systems to combat free radical damage [4,26]. However, previous work from this laboratory has shown that aging leads to many oxidative changes in the adrenal including a dramatic reduction in the normally protective antioxidant defense system [4], which probably lead to the decline of corticosterone production in aging animals [27,28].

Our current thinking is that during aging, cumulative oxidative damage occurs at the levels of transcription/translation, and ultimately affects the function of protein factors necessary for intracellular cholesterol processing and transport to mitochondrial sites of P450scc. Indeed, we and others have already shown that functional expression of steroidogenic acute regulatory (StAR) protein and peripheral-type benzodiazepine receptors (PBR), the two key proteins involved in cholesterol transport, are greatly reduced during aging [11,12]. Additional evidence comes from our demonstration that expression of "stress responsive" transcription factors AP-1 [29–31] and nuclear factor–κB (NF-κB) [30,32,33] are both substantially reduced in adrenal extracts from aging animals [27,28].

The purpose of this study was to more directly examine the long-term chronic effects of oxidative stress, as it occurs during aging, on the functional expression of oxidant sensitive AP-1 transcription factor in rat adrenals. We chose to study adrenal gland for the following reasons: AP-1 is constitutively active in this tissue; its expression does not require pretreatment with stressors; adrenal AP-1 activity is reduced during aging [27]; and AP-1 regulates the expression of several key proteins that are crucial for steroidogenesis [27,28]. For these studies, in vivo chronic oxidative stress and lipid peroxidation were experimentally induced in rats by feeding them a diet deficient in vitamin E [34,35]. Vitamin E is a family of naturally occurring and lipid-soluble compounds, the tocopherols and the tocotrienols, of which α-tocopherol is the most biologically active antioxidant in vivo [36]. It is sequestered in the hydrophobic interior of membranes, where it acts as an important chain-breaking antioxidant by quenching lipid peroxidation, and helps to maintain the integrity of biological membranes [36,37]. We investigated the mechanism of this regulation with respect to levels of AP-1 constituent proteins (Fos/Jun family proteins) and redox factor-1 (Ref-1, also known as APE, HAP, and APEX), which functions as both a nuclear DNA repair enzyme and as a reversible regulator of the DNA-binding activity of several transcription factors, including AP-1 by altering the redox state of specific cysteine residues located in the basic DNA binding region of these transcription factors [38–40].

Results obtained from the current study indicate that vitamin E deficiency–induced chronic oxidative stress significantly down-regulates adrenal AP-1 activity. Moreover, AP-1 inhibition seems to be due to both reduced levels of AP-1 constituent proteins as well as Ref-1. Based on the evidence presented here, we suggest that oxidative stress/free radical–mediated inhibition of adrenal AP-1 activity may represent a common mechanism resulting in the reduced functional efficiency of key proteins involved cholesterol transport and its conversion to steroids as observed during aging.

2. Methods and materials

2.1. Reagents

[γ-^{32}P] ATP (3000 Ci/mmol, 111 TBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). T4 polynucleotide kinase, kinase reaction–buffer and double-stranded oligonucleotides that represent consensus sequences to AP-1, SP-1, NF-κB transcription factors were obtained from Promega Corp. (Madison, WI). The following chemicals were supplied by Sigma Chemical Co. (St. Louis, MO): thiobarbituric acid, α-tocopherol, ascorbic acid, Bt_2cAMP, corticosterone, β-nicotinamide adenine dinucleotide phosphate (βNADPH), spermidine, spermine, pepstatin A, leupeptin, aprotenin, paramethyl sulfonyl fluoride (PMSF), dithiothreitol, and sucrose. The affinity-purified rabbit polyclonal immunoglobulin G antibodies (IgG) against c-Fos, c-Jun, JunB, JunD, Fra-1, Fra-2, and FosB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies were used for Western blotting and/or gel shift analysis and were mouse, rat, and human reactive. ACTH (Cortrosyn) was obtained from Organon, Inc. (West Orange, NJ). Anticorticosterone antibody was the product of Endocrine Sciences (Tarzana, CA). All other reagents used were of analytical grade.

2.2. Animals, experimental design, and tissue collection

Male, Sprague-Dawley rats (n = 32; initial weight, 87–129 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed two animals per cage covered with a microisolator in a room controlled for humidity, temperature (21° ± 1°C), and light (12-hour light:dark cycle). Rats were acclimated for 48–72 hours and then randomly divided into two dietary groups (n = 16/group) with adjustments made to keep mean group weights comparable. Group A received a control (vitamin E–adequate) diet, whereas group B received a diet totally deficient in vitamin E. Both diets were provided by Harlan Teklad (Madison, WI). The composition of both diets were identical except that the vitamin E–adequate diet contained 0.15 g of vitamin E acetate (500 IU/g)/kg diet, whereas the deficient diet contained no vitamin E. The rats were maintained on these diets for a period of 5–6 months, and fed ad libitum with unrestricted access to drinking water. No overall differences in food consumption were noted between animals fed the deficient or control diet. The rats were weighed once each week and at the end of the study.

At the end of the feeding period, rats were killed by decapitation and blood collected in heparinized tubes for the isolation of plasma and quantification of plasma–associated α-tocopherol. Liver, adrenal, testis, and, if required, other tissues (e.g., heart, brain, lung, and kidney) were immediately removed, rinsed in phosphate-buffered saline, blotted, and weighed. The tissues were either processed immediately for the isolation of cells, and subcellular fractions or stored frozen at −80°C until analyzed for AP-1 activity, AP-1 protein constituents, or tissue vitamin E content.

2.3. Isolation of adrenocortical cells and in vitro steroidogenesis

Freshly isolated adrenocortical cells [7] from control and vitamin E–deficient rats were used to assay steroidogenesis. Cell samples were incubated with ±ACTH (10 ng/mL), ± Bt_2cAMP (2.5 mmol/L), or ±20α-hydroxycholesterol (10 μmol/L) at 37°C for 3 hours, and samples of incubation medium were assayed for corticosterone by direct radioimmunoassay as described previously. Results represent nanograms of corticosterone produced per microgram of DNA and are expressed as the mean ± SE of duplicate determinations of four different adrenal cell preparations derived from four individual animals.

2.4. Measurement of lipid peroxidation

Tissue susceptibility to lipid peroxidation was quantified by colorimetric determination of thiobarbituric acid-reactive substances (TBARS) as described previously [4,41]. Aliquots of adrenal membrane preparations (100–120 μg of protein) were incubated at 37°C for 60 minutes in 50 mmol/L Tris-maleate buffer, pH 7.4 containing 0.1 mol/L KCl (1 mL total volume) in the absence (basal) or presence of either 130 μmol/L ascorbate and 26 μmol/L ferrous sulfate [42] (nonenzymatic) or 1 mmol/L NADPH, 2 mmol/L ADP and 20 mmol/L $FeCl_3$(enzymatic) [43]. After incubation, the peroxidation reaction was stopped by rapid addition of 0.2% butylated hydroxy toluene (50 μL), followed by 20% trichloroacetic acid (0.3 mL) and 50 mmol/L thiobarbituric acid (0.6 mL) [41]. The samples were mixed thoroughly and placed in a boiling water bath for 8 minutes. The resulting chromagen in each case was extracted with a mixture of *n*-butanol:water:pyridine (15:3:1 v/v) and absorbances of organic phase were simultaneously determined at 510, 532, and 560 nm [44]. The amount of 532 nm absorption resulting from TBARS (malondialdehyde, MDA equivalent) was calculated by the following equation [44], which uses an extinction coefficient of 1.56×10^{15} cm^{-1} for MDA: $MDA_{532} = 1.22\ [(A_{532}) - (0.56)\ (A_{510}) + (0.44)\ (A_{560})]$.

2.5. Preparation of nuclear extracts and electrophoretic mobility shift assays

Adrenal nuclear extracts were prepared as described previously from this laboratory [27]. The double-stranded oligonucleotide probes were end-labeled using [γ-^{32}P] ATP and T_4 polynucleotide kinase, and unincorporated radioactivity in each preparation was removed by Sephadex G-25 spin column chromatography (Roche Diagnostics Corp., Indianapolis, IN). Electrophoretic mobility shift assays (EMSAs) were carried out as previously described with minor modifications [27,28]. Briefly, adrenal nuclear extracts (2.0–2.5 μg protein) were incubated with ^{32}P-labeled double-stranded oligonucleotide probe (50,000–100,000 DPM) in a 20-μL reaction mixture for 20 minutes at room temperature. Each reaction mixture for AP-1 contained the following: 15 mmol/L HEPES-NaOH (pH 7.9), 3 mmol/L Tris-HCl (pH 7.9), 60 mmol/L KCl, 0.5 mmol/L EDTA, 1 mmol/L $MgCL_2$, 100 μg/mL poly (dI-dC).poly (dI-dC),0.5 mmol/L DTT, 1% NP-40, and 10% glycerol. For SP-1 the mixture contained the following: 50 mmol/L Tris-HCl (pH 7.9), 100 mmol/L KCl, 12.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 100 μg/mL poly (dI-dC).poly (dI-dC), 1 mmol/L DTT, 1% NP-40, and 10% glycerol. The 32P-oligonucleotide-nuclear protein complexes formed were separated from free oligonucleotides by electrophoresis through a 4% native polyacrylamide gel in running buffer of 0.5 × TBE at 150 V (~40 mA) for 2–3 hours until bromophenol blue was ~1–2 cm from bottom of gel. Gels were dried and exposed to x-ray (Kodak X-Omat, Rochester, NY, USA) film for 5–20 hours at room temperature, and were then scanned and the appropriate bands quantified by densitometry. The upper strand of the synthetic oligonucleotides containing AP-1, NF-κB, SP-1 recognition sequences (the consensus sequences shown in boldface type) were as follows: AP-1 (TRE)—5′-CGC T**TG ATG AGT** CAG CCG GAA-3′ SP-1—5′-ATT CGA T**CG GGG CGG GG**C GAG C-3′

Competition experiments were performed by preincubating the extracts with unlabeled AP-1, SP-1, or NF-kB oligonucleotides for 5 minutes at room temperature before the addition of ^{32}P-labeled oligonucleotide probe, and were followed by incubation at room temperature for an additional 20 minutes.

Composition of AP-1 DNA-binding complexes was determined by performing antibody supershift and antibody inhibition assays. Adrenal nuclear extracts were preincubated with 2 μg of polyclonal rabbit antibodies specific for the Fos and Jun protein families or preimmune rabbit IgG for 60 minutes at 4°C before the DNA-protein binding reaction in EMSA. The DNA-protein complexes were subsequently resolved by electrophoresis as described above.

2.6. Western blotting

Adrenal nuclear protein extracts were prepared as described above. Equal amounts of nuclear fractions (30–40

μg of protein) were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to Immobilon-P membranes as described earlier [27,28]. After staining with Ponceau S to verify loading equivalency and transfer efficiency [27,28], the membranes were incubated with anti–c-Fos, FosB, Fra-1, Fra-2, JunD, c-Jun, JunB, or Ref-1 followed by treatment with horseradish peroxidase–conjugated goat antirabbit IgG. Bands were visualized by enhanced chemiluminescence detection as described by the manufacturer (ECL System, Amersham/Pharmacia Biotech, Arlington Heights, IL, USA). Blots were exposed to film for various times (3–10 minutes), and exposures were subjected to densitometric scanning.

2.7. Analytical methods

The concentrations of α-tocopherol in plasma, liver, adrenal, and other tissues were measured by the high-performance liquid chromatography method as previously described [4]. The protein concentrations of membrane fractions were determined by the colorimetric procedure of Markwell et al. [45], and the modified Lowry procedure of Peterson [46] was used to quantify protein content in nuclear extracts.

2.8. Statistical analysis

The Student *t* test was used for statistical evaluation of the results. A P value of < 0.05 was considered to be significant. The program used for statistical analysis was GRAPH PAD PRISM™, version 3.0 (Graph Pad Software, San Diego, CA).

3. Results

Table 1 shows body weights and levels of α-tocopherol in plasma, liver, adrenal, and testicular tissues of rats fed either a vitamin E–adequate (control) diet or vitamin E–deficient diet. At the beginning and end of the 6-month study, the overall body weights were not significantly different between the control and vitamin E–deficient groups of rats. Also, food intake was not affected by vitamin E deficiency (data not shown). Vitamin E deprivation of rats significantly decreased α-tocopherol concentration in plasma, liver, adrenal gland, and testis and the extent of the reduction in α-tocopherol concentrations were comparable (90–95%) among the three tissues (Table 1). However, as reported before, the α-tocopherol levels of control adrenals were 10- to 12-fold greater than liver values.

TBARS measurements were made to assess the extent of lipid peroxidation in adrenal and liver membrane preparations from rats maintained on vitamin E–adequate (control) or vitamin E–deficient diets for 6 months. These measurements were made both under basal conditions and after exposure to either enzymatic (Fe^{2+}/ADP/NADPH) or nonenzymatic (Fe^{2+}/ascorbate) initiators. Consistent with the previous data [4,34,35], adrenal membranes from control animals displayed only minimal endogenous levels of TBARS and only an approximately 10-fold increase in malondialdehyde levels after exposure to either enzymatic or nonenzymatic initiator. Moreover, adrenal membranes were more resistant to lipid peroxidation than the liver under ascorbate-induced and conditions induced by reduced nicotinamide adenine dinucleotide phosphate (NADPH), as well as lipid peroxidation without added initiators (Table 2). In contrast, adrenal membrane preparations from vitamin E–deficient rats were more susceptible to lipid peroxidation than that of the controls and exhibited roughly 10-fold higher basal levels and approximately 20- to 25-fold higher TBARS levels than control in response to prooxidants. Finally, the TBARS content of vitamin E–deficient adrenal membranes in response to either enzymatic or nonenzymatic initiator was comparable to the values obtained with liver membranes from both control and vitamin E–deficient animals.

Table 1
Body weight, and plasma, liver, and adrenal vitamin E content of rats fed either a control (adequate vitamin E) or a vitamin E–deficient diet

	Control diet	Vitamin E–deficient diet	P
Body weight			
Initial (g)	106 ± 2	107 ± 3	
Final (g)	540 ± 11	529 ± 13	= NS
Plasma vitamin E (μg α-tocopherol/mL)	6.8 ± 1.2	0.34 ± 0.07	< 0.0005
Liver vitamin E (μg α-tocopherol/g liver)	16.7 ± 3.1	0.76 ± 0.17	< 0.0001
Adrenal vitamin E (μg α-tocopherol/g adrenal)	207 ± 18	20.3 ± 7.2	< 0.001
Testis vitamin E (μg α-tocopherol/g testis)	25.5 ± 6.7	0.93 ± 0.25	< 0.0005

Values (mean ± SE) are expressed as μg equivalent of α-tocopherol/g tissue except plasma (μg/mL) (n = 14–16 animals/group). Feeding rats a vitamin E–deficient diet for approximately 5½ months reduced plasma levels of vitamin E by 95% and dramatically reduced the levels of vitamin E in liver, adrenal, and other organs (e.g., brain, heart, lung, kidney, spleen and testis; data not shown).

The effects of vitamin E deficiency on ACTH- and Bt_2CAMP-induced or 20 α-hydroxycholesterol–supported secretion of corticosterone by isolated adrenocortical cells are shown in Table 3. These data demonstrate that the mean basal level of corticosterone in adrenal cells from control rats was 5.3 ng/μg DNA compared to 1.8 ng/μg DNA in vitamin E–deficient rats. Maximal ACTH-induced corticosterone production also diminished with vitamin E deficiency, with mean corticosterone levels in the adrenocortical cells from control rats of 187 ng/μg DNA compared with 96 ng/μg DNA from vitamin E–deficient rats. Likewise, Bt_2cAMP-induced corticosterone production also de-

Table 2
Basal and pro-oxidant-induced lipid peroxidation levels in adrenal and liver of rats fed either a vitamin E adequate control diet or a diet deficient in vitamin E.

Tissue	Pro-oxidants	nmol MDA produced/60 min/mg protein ± SE	
		Control diet	Vitamin E–deficient diet
Adrenal			
	None	0.08 ± 0.01	0.94 ± 0.15*
	Fe^{2+}/ADP/NADPH	1.11 ± 0.20	24.2 ± 3.1
	Fe^{2+}/ascorbate	1.17 ± 0.33	27.7 ± 5.3
Liver			
	None	0.67 ± 0.14	13.6 ± 1.9†
	Fe^{2+}/ADP/NADPH	23.5 ± 3.1	27.6 ± 5.3
	Fe^{2+}/ascorbate	19.1 ± 2.2	24.4 ± 4.4

Results are mean ± SE of six separate experiments. These results that vitamin E deficiency causes adrenal and liver tissues to excessive lipid peroxidation especially under basal conditions (i.e., in the absence of externally added pro-oxidants)

* $P < 0.001$ (control *vs* deficient diet).

† $P < 0.01$ (control *vs* deficient diet).

creased in response to vitamin E deficiency. Thus, the maximal concentration of corticosterone in adrenocortical cells of control rats was 225 ng/μg DNA compared with 159 ng/μg DNA in the vitamin E–deficient group.

In an attempt to determine whether the adrenal steroidogenic pathway is compromised by vitamin E deficiency–induced oxidative stress, we also measured 20 α-hydroxycholesterol–supported corticosterone secretion (Table 3). The 20 α-hydroxy-cholesterol, like other hydroxycholesterols, is freely transported to michondria, and the extent of its conversion into steroids is indicative of the functional efficiency of the steroidogenic pathway. Adrenocortical cells isolated from rats fed a control diet showed robust secretion of corticosterone when challenged with 20 α-hydroxycholesterol. However, 20 α-hydroxycholesterol–supported responses were significantly decreased in adrenal cells from vitamin E–deficient rats. These results suggest that vitamin E deficiency–induced chronic oxidative stress not only inhibits steroidogenesis but also negatively impacts the efficiency of the steroidogenic pathway.

Table 3
Corticosterone production by isolated adrenocortical cells from rats fed a normal diet or a diet deficient in vitamin E

Additions	Corticosterone (ng/μg DNA	
	Control diet	Vitamin E–deficient diet
Basal (no addition)	5.3 ± 0.9	1.8 ± 0.4*
ACTH (10 ng/mL)	187.0 ± 21.1	96.3 ± 8.9†
Bt_2cAMP (2.5 mmol/L)	225.3 ± 24.3	159.2 ± 25.6‡
20 α-Hydroxycholesterol (20 μmol/L)	297.0 ± 26.0	173.1 ± 20.0§

Results are mean ± SE of eight separate experiments.

* $P < 0.003$; † $P < 0.001$; ‡ $P < 0.005$; § $P < 0.01$.

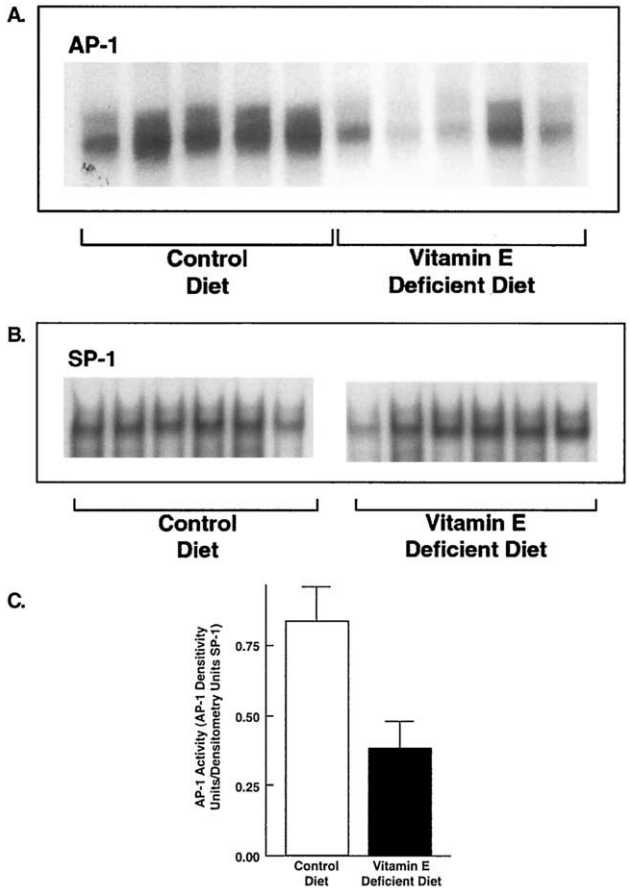

Fig. 1. EMSA demonstrating AP-1 and SP-1 binding activities in the adrenal nuclear extracts isolated from rats fed a control diet or a diet deficient in vitamin E. (*A*) EMSA of AP-1 binding activity. Nuclear extracts (2 μg protein) were incubated with [^{32}P] labeled-AP-1 TRE oligonucleotide probe as described in the "Methods and materials" section. (*B*) EMSA of SP-1 DNA-binding activity. Adrenal nuclear extracts were analyzed by EMSA for [^{32}P] labeled SP-1 oligonucleotide binding as described in the "Methods and materials" section. (*C*) Numerical values demonstrating the effects of vitamin E deficiency on AP-1 DNA-binding activity. Values were derived from results similar to those shown in *A* and *B*. All AP-1 binding data were normalized to SP-1 DNA-binding activity and are expressed as the mean ± SE of five different experiments. ($P < 0.05$.)

To correlate cellular responses with molecular events, we next investigated the effects of vitamin E deficiency and resultant oxidative stress on the activities of oxidant sensitive transcription factors, AP-1 and NF-κB. To assess AP-1 activity, EMSAs were performed using a probe containing the 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE) consensus sequence, $TGA^{C}/_{G}TA$ (1,69). As shown in Fig. 1A, adrenal nuclear extracts from control rats fed a vitamin E–adequate diet contained one major and one minor constitutively active AP-1 binding complex. These results are in agreement with previous studies demonstrating the constitutive expression of AP-1 DNA-binding activity in rat adrenals [27,47]. Likewise, adrenal nuclear extracts from rats maintained on a vitamin E–deficient diet also exhibited a single prominent band and a minor band, which migrated

with similar mobility to that seen in control adrenals. However, vitamin E deficiency resulted in a significant decrease (50–60%) in adrenal AP-1 DNA-binding activity. To determine whether the vitamin E–induced loss in AP-1 activity was specific, the binding activity of SP-1 was also evaluated. Under similar experimental conditions, the binding activities of SP-1 (Fig. 1B) did not show any significant change in response to vitamin E depletion. Figure 1C summarizes and quantifies the data presented in Fig. 1A and 1B. Adrenal AP-1 binding activities were evaluated by scanning densitometry to allow quantitative comparison between control and vitamin E–deficient animals. To ensure that the vitamin E–induced alteration in AP-1 binding was not due to aberrant protein loading or extraction bias, all results were normalized to SP-1 activity. These analyses further confirmed that vitamin E deficiency is accompanied by a significant reduction (~55%) in AP-1 DNA-binding activity (Fig. 1C).

AP-1 is composed of either homodimers of Jun family members (c-Jun, Jun B, Jun D) or heterodimers of Jun proteins with Fos family members (c-Fos, Fos B, Fra 1, and Fra 2) [29,31]. Considerable evidence suggests that dimeric composition is an influential determinant of the AP-1 binding and transactivational potential [29,31]. Therefore, to delineate whether the reduced expression of AP-1 in adrenals of vitamin E–deficient rats was due to alteration of the dimeric composition of AP-1 homo-/heterdimers, we compared the subunit composition of AP-1 complexes in adrenal nuclear extracts from rats fed a control diet or a diet deficient in vitamin E. To identify the Fos and Jun families that participate in AP-1 complex formation, polyclonal antisera against the individual c-Fos and Jun proteins were added to the EMSA reaction. The inhibition, or altered electrophoretic mobility, of complex formation by specific antiserum is taken as the presence of that protein in the oligonucleotide probe–transcription factor complex. As shown in Fig. 2, the antibodies to Jun D and Fos B "supershifted" the AP-1 specific DNA complexes in adrenal extracts from both the control and vitamin E–deficient groups. However, antibodies to c-Fos, c-Jun, Jun B, Fra 1, and Fra 2 had no discernable effect on the AP-1 binding in either of the two groups. From these data we concluded there were no major differences in the subunit composition of the AP-1 complexes between the control and vitamin E–deficient groups.

We next evaluated whether the vitamin E deficiency induced attenuation of AP-1 activity was correlated with a reduction in the levels of Jun and Fos families of proteins. Adrenal nuclear extracts from control and vitamin E–deficient adrenals were subjected to Western blot analysis using antibodies directed against the Jun and Fos families of proteins (Fig. 3). The quantitative data obtained from six individual blots are presented in Table 4. As can be seen, vitamin E deficiency significantly reduced the steady state levels of all members of Fos and Jun family members with the exception of Fra 1 and Jun B, the levels of which were

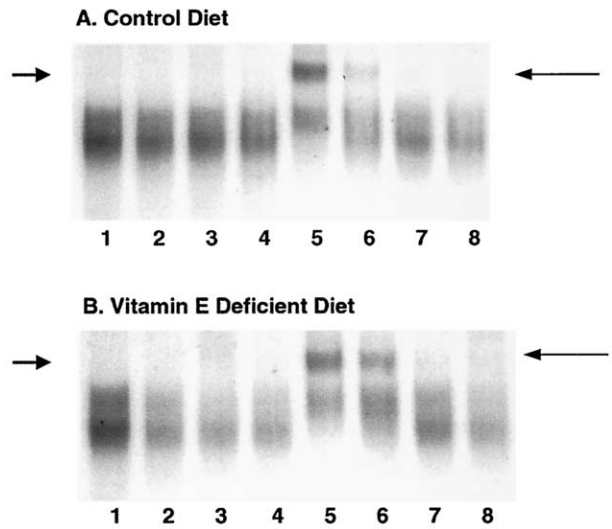

Fig. 2. Super-shift/inhibition analysis of the dimeric composition of the AP-1 DNA-binding activities in adrenal nuclear extracts from rats maintained on a vitamin E adequate (control) or vitamin E–deficient diet. Nuclear extracts (2 μg of control or 4 μg vitamin E–deficient) were pretreated with vehicle (preimmune IgG) (lane 1), anti–c-Fos (lane 2), anti–c-Jun (lane 3), anti–Jun B (lane 4), anti–Jun D (lane 5), anti–Fos B (lane 6), anti–Fra 1 (lane 7), or anti–Fra 2 (lane 8) at 4°C for 60 minutes and EMSAs were performed. The arrows show the DNA binding complexes supershifted by the antibodies.

below the detection limits of antibodies used and thus could not be accurately measured.

Another potential mechanism by which AP-1 DNA-binging activity may be regulated is through changes in intracellular oxidation/reduction reactions involving the redox factor-1 (Ref-1) protein. Ref-1, also known as APE-1/Hap-1/APEX is a ubiquitously expressed, bifunctional nuclear enzyme that acts as an endonuclease in the base excision repair of oxidatively modified DNA and as an agent reducing protein to modulate the oxidation/reduction (redox) state of specific cysteine residues, which promotes the DNA binding activities of many transcription factor including AP-1 [38,39,48,49]. Therefore, we sought to determine whether Ref-1 played a role in the vitamin E deficiency–induced decrease in AP-1 DNA-binding activity. Using Western blot analysis, we quantified Ref-1 protein levels in adrenal nuclear extracts from rats fed either a control diet or a diet deficient in vitamin E. Ref-1 protein expression was significantly reduced in adrenals of vitamin E–depleted animals (Fig. 4). Scanning densitometry analysis of the immunopositive bands demonstrated that vitamin E deficiency reduced Ref-1 protein content by ~50% ($P < 0.0242$), as shown in Fig. 5.

4. Discussion

Increasing evidence suggests that elevated levels of cellular oxidative stress contribute to aging [13–16]. High

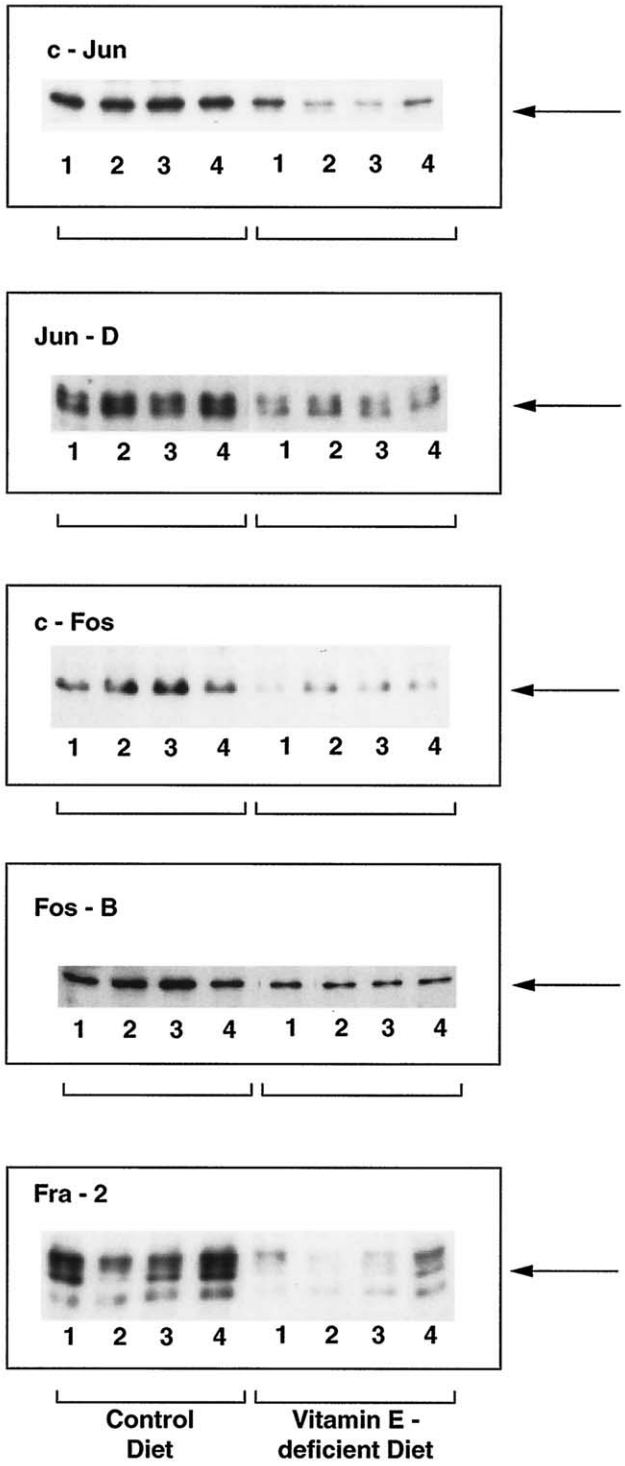

Fig. 3. Immunoblot analysis of c-Jun, Jun-D, c-Fos, Fos-B, and Fra-2 proteins in adrenal nuclear extracts prepared from rats on control or vitamin E–deficient diets as described in the "Methods and materials" section. This figure is representative of four separate experiments and the densotometric quantitation is presented in Table 4.

Table 4
Scanning densitometry of the c-Fos and c-Jun family of proteins in adrenal nuclear extracts from rats fed a control diet or a vitamin E–deficient diet

	Arbitrary Units/Unit Protein ± SE		
	Control diet	Vitamin E-deficient diet	P
c-Fos	19.42 ± 2.0	8.39 ± 1.4	<0.005
c-Jun	42.25 ± 1.2	35.97 ± 2.0	<0.03
Jun D	46.81 ± 2.9	31.32 ± 1.6	<0.001
Fos B	24.15 ± 1.8	17.59 ± 0.8	<0.05
Fra-2	86.41 ± 6.0	52.66 ± 4.7	<0.005
Fra-1	BD	BD	

Results are mean ± SE of four separate experiments.
BD = below detection limit.

levels of ROS may damage DNA [17], cause degradation of membrane lipid constituents due to enhanced lipid peroxidation [18], and inactivate proteins [19], resulting in chronic dysfunction of vital cellular processes. The steroidogenic tissues are particularly vulnerable to the deleterious actions of oxidative stress because they contain a high content of unsaturated membrane lipids, use excessive amounts of molecular oxygen for steroid synthesis, and generate ROS (e.g., oxygen-centered free radicals and peroxides) as by-products of oxidative metabolism [4,20,50–53]. Lipid peroxidation is a membrane degradative process arising as a consequence of the production and propagation of free radical reactions, and can therefore modulate membrane structure and/or fluidity. Because almost every event associated with cholesterol mobilization as well as transport to and use by mitochondria is dependent on the integrity of cellular membranes [23–25], the chances of steroidogenesis being adversely affected are quite high in tissues subjected to chronic oxidative stress. As a result, steroidogenic tissues are especially well equipped with a variety of antioxidant defenses including reduced glutathione, glutathione peroxidase, glutathione reductase, catalase, ascorbate, superoxide dismutases (SOD, Cu/Zn-SOD, and Mn-SOD) and vitamin E (α-tocopherol), which protect the cells from the deleterious actions of free radicals (e.g., superoxide, hydrogen peroxide, and lipid peroxidation reactions) [4,26].

Our laboratory has recently demonstrated that antioxidant protection is greatly compromised during aging and

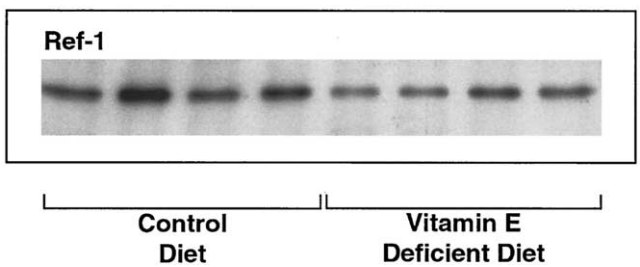

Fig. 4. Levels of Ref-1 in adrenal nuclear extracts from control and vitamin E deficient rats. Adrenal nuclear extracts were separated on 10% SDS-PAGE gels, transferred to nylon membranes, and immunoblotted with anti–Ref-1 as described in the "Methods and materials" section. This figure is representative of four experiments.

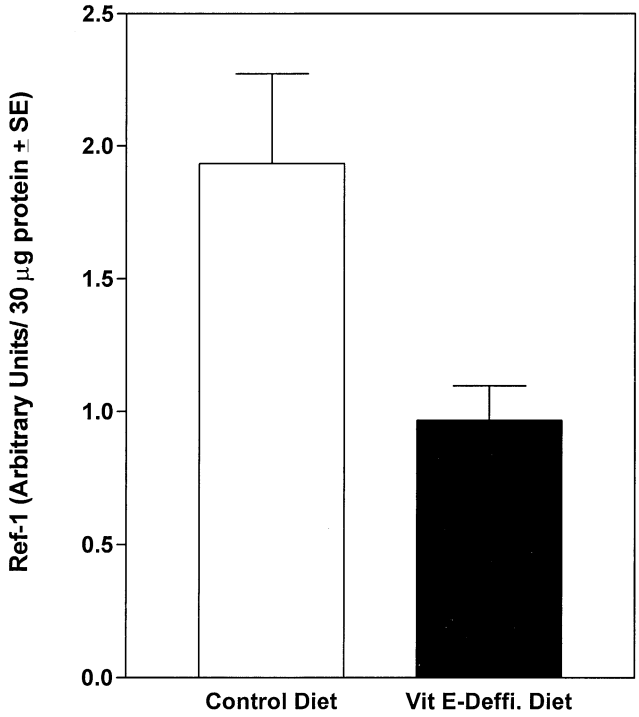

Fig. 5. Numerical values demonstrating the effects of vitamin E deficiency on Ref-1 expression in rat adrenal gland. Values were derived from results similar to those shown in Fig. 4. Results are mean ± SE of six separate experiments.

that, as a consequence, adrenals of aged rats exhibit markedly elevated levels of oxidative stress and enhanced sensitivity to lipid peroxidation, which ultimately lead to the decline of corticosterone production in aging animals [4]. Moreover, the deleterious effects of oxidative stress appear to affect three major cellular processes. One such process is the adrenal antioxidant defense system itself. Indeed, the steady levels of Cu-Zn SOD, Mn SOD, GPX, and vitamin C as well as reduced glutathione are greatly decreased in adrenals of aging rats, while at the same time the susceptibility of adrenal membranes to lipid peroxidation is profoundly enhanced [4]. A second process that is severely impaired during aging is the functional expression of StAR and PBR, the key proteins involved in cholesterol transport to and within the mitochondrial sites of CYP11A1 (P450scc) [11,12]. Finally, impaired expression of oxidant sensitive transcription factors, AP-1, and NF-κB, has also been observed in adrenals of aging rats [27,28]. Thus, a correlation between the loss of steroidogenic response and oxidative stress–induced impairment of StAR, PBR, AP-1, and NF-κB in aging adrenals, in conjunction with accumulating evidence that oxidative stress is a major contributor to aging process, raises the possibility that oxidative stress may contribute to the age-related decline in steroid hormone synthesis by rat adrenals.

To explore potential cellular mechanism that may confer transcriptional susceptibility to oxidative stress, the current studies were initiated to examine the impact of depletion of vitamin E on the functional expression of the oxidative stress–responsive transcription factor AP-1. Vitamin E is the major lipid-soluble, chain-breaking antioxidant that is sequestered in the hydrophobic interior of membranes, where it functions as an important quencher of lipid peroxidation and helps to maintain the integrity of biological membranes [36,37]. Loss of cellular levels of vitamin E is known to cause excessive oxidative stress, lipid peroxidation, and oxidative damage in vivo [36,37]. We report that vitamin E deficiency, achieved by feeding a diet deficient in vitamin E, resulted in the inhibition of AP-1 DNA-binding activity with concomitant down-regulation of corticosterone production. Vitamin E deficiency–induced oxidative stress appeared to inhibit AP-1 DNA-binding activity by reducing the steady state levels of constituent Fos and Jun families of proteins as well as the redox-regulated protein, Ref-1. The current work confirms that vitamin E deficiency induces increased lipid peroxidation [34,35] and consequently leads to inhibition of steroidogenesis. In addition, the use of a freely diffusible steroid precursor, 20α-hydroxycholesterol, demonstrated that vitamin E deficiency–induced chronic oxidative stress appears to interfere not only with cholesterol transport to mitochondria but also to affect the steroidogenic pathway responsible for the conversion of precursor cholesterol into steroids. Our interpretation of these results is as follows: because CYP11A1 (P450scc), CYP11B1 (P45011β), CYP21 (P450c21) are all cytochrome P450 requiring enzymes, excessive oxidative stress inhibits one or more of these enzymes. This suggestion is based on the previous observations that cytochrome P450 requiring enzymes are highly susceptible to oxidative insult [50,51,54,55], and that some of these hydroxylases are down-regulated during aging [5]. Additionally, StAR protein, which facilitates cholesterol transport to mitochondria, likely is vulnerable to inhibition by oxidative stress because StAR mRNA and protein levels are significantly reduced during aging [11,56]. Thus, the deleterious actions of oxidative stress on steroidogenesis are likely to involve effects on both cholesterol mobilization and transport to mitochondria as well as its conversion to steroid products.

AP-1 is a heterodimeric complex composed of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families of proteins, as either Jun:Jun homodimers or Jun:Fos heterodimers [29,31]. Various combinations of these two families of proteins dictate the extent and outcome of AP-1 binding [29,31]. In this study we observed no alterations in specific members of the Jun and Fos families by vitamin E deficiency–induced oxidative stress. Supershift analyses indicated that JunD and FosB are the major constituents of AP-1 in adrenal nuclear extracts obtained from rats maintained on either vitamin E–adequate or vitamin E–deficient diets. Other proteins such as c-Fos, c-Jun, JunB, Fra1, and Fra2 also contribute to varying extent in AP-1 complex formation and, again, their overall contributions were not influenced by vitamin E depletion. The in-

hibition of constitutively active AP-1 binding by vitamin E deficiency is at least in part due to suppression of Fos and Jun proteins. Indeed, we have determined by Western blotting that vitamin E deficiency elicits decreases in the levels of all members of the Jun/Fos families. Our data are consistent with previous studies, showing that major adrenal AP-1 constituents are drastically reduced during aging [27]. In addition, although not tested directly, there is a possibility of a direct effect of oxidative stress on posttranslational modifications, especially the phosphorylation state of the AP-1 family members [29–31].

Vitamin E deficiency–induced oxidative stress also results in down-regulation of Ref-1. Ref-1 is a ubiquitously expressed bifunctional 36 kDa protein that acts as a DNA repair enzyme, as well as, a stimulator of DNA-binding activity by several transcription factors, including AP-1, through a redox-dependent mechanism [38,39,48,49]. Reduced Ref-1 expression in vitamin E–deficient adrenals is likely to have multiple functional consequences in addition to impaired genomic repair. Among these, lack of Ref-1 could affect the redox state of many transcription factors including AP-1 that may affect the functional expression of many genes that participate in stress signaling [30,40] and steroidogenic pathways [27]. Also, optimal expression of Ref-1 is required for proper mitochondrial function [57,58]. Because mitochondria are also the primary site for steroid hormone production, reduced Ref-1 expression may affect overall steroidogenic capacity of the adrenal gland. Moreover, Ref-1 expression is subject to hormonal regulation [59,60], and reduced expression of Ref-1 along with AP-1 could seriously compromise the functional efficiencies of steroidogenic cell proteins (e.g., CYP11B1, trophic hormone receptors, StAR protein). Thus, impaired expression of AP-1/Ref-1 is likely a major contributor to oxidative stress–induced loss of steroid hormone production.

In summary, the key findings of our work are that vitamin E deficiency–induced oxidative stress leads to decreased expression of Jun and Fos families of proteins as well as inhibition of constitutively active AP-1 DNA-binding by a mechanism that also appears to involve Ref-1 protein. These results support the concept that chronic oxidative stress exerts a negative effect on oxidant-responsive AP-1 DNA-binding activity, which in turn leads to inefficient functional expression of crucial steroidogenic and cholesterol transport proteins and concomitant reduction in steroidogenic response.

Acknowledgments

This work was supported in part by Office of Research Development, Medical Research Service, Department of Veterans Affairs, and by a grant DK56339 from the National Institutes of Health.

References

[1] Nowak FW, Mooradian AD. Endocrine function and dysfunction. Encycl Gerontol 1996;1:477–91.
[2] Lamberts SWJ, van den Beld AW, van der Lely A-J. The endocrinology of aging. Science 1997;278:419–24.
[3] Burger HG, Dudley EC, Robertson DM, Dennerstein L. Hormonal changes in the menopause transition. Recent Prog Horm Res 2002; 57:257–75.
[4] Azhar S, Cao L, Reaven E. Alteration of the adrenal antioxidant system during aging in rats. J Clin Invest 1995;96:1414–24.
[5] Zirkin BR, Chen H. Regulation of Leydig cell steroidogenic function during aging. Biol Reprod 2000;63:977–81.
[6] Wang C, Hikim AS, Ferrini M, Bonavera JJ, Vernet D, Leung A, Lue YH, Gonzalez-Cadavid NF, Swerdloff RS. Male reproductive ageing: using the brown Norway rats as a model for man. Novartis Found. Symp 2002;242:82–95.
[7] Popplewell PY, Azhar S. Effects of aging on cholesterol content and cholesterol-metabolizing enzymes in the rat adrenal gland. Endocrinology 1987;121:64–73.
[8] Liao C, Reaven E, Azhar S. Age-related decline in the steroidogenic capacity of isolated rat Leydig cells: a defect in cholesterol mobilization and processing. J Steroid Biochem 1993;46:39–47.
[9] Azhar S, Reaven E. Effect of age on cholesterol uptake and utilization by rat adrenals: Internalization of lipoprotein-derived cholesteryl esters. Mech Ageing Dev 1994;77:13–25.
[10] Cheng B, Chou SC, Abraham S, Kowal J. Effects of prolonged ACTH-stimulated on adrenocortocal cholesterol reserve and apolipoprotein E concentration in young and aged Fischer 344 male rats. J Steroid Biochem Biol 1998;66:335–45.
[11] Leers-Sucheta S, Stocco DM, Azhar S. Down-regulation of the steroidogenic acute regulatory (StAR) protein in rat Leydig cells: implications for regulation of testosterone production during aging. Mech Ageing Dev 1999;107:197–203.
[12] Culty M, Luo L, Yao ZX, Chen H, Papadopoulos V, Zirkin BR. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. J Androl 2002;23:439–47.
[13] Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1957;2:298–300.
[14] Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998;78:547–81.
[15] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239–47.
[16] Drew B, Leeuwenburgh C. Aging and the role of reactive nitrogen species. Ann. N . Acad Sci 2002;959:66–81.
[17] Gilchrest BA, Bohr VA. Aging processes, DNA damage, and repair. FASEB J 1997;11:322–30.
[18] Mylonas C, Kouretas D. Lipid peroxidation and tissue damage. In Vivo 1999;13:295–309.
[19] Stadtman ER. Protein oxidation in aging and age-related diseases. Ann NY Acad Sci 2001;928:22–38.
[20] Hornsby PJ. Steroid and xenobiotic effects on adrenal cortex: mediation by oxidative and other mechanisms. Free Radic Biol Med 1989;6:103–15.
[21] Cadenas E, Davies KJA. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 2000;29:222–30.
[22] Raha S, Robinson BH. Mitochondria, oxygen free radicals, disease and aging. TIBS 2000;25:502–8.
[23] Stocco DM, Clark BJ. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. Biochem Pharmacol 1996;51:197–205.
[24] Papadopoulos V, Amri H, Boujard N, Cascio C, Culty M, Garnier M, Hardwick M, Li H, Vidic B, Brown AS, Reversa JL, Bernassau JM, Drieu K. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. Steroids 1997;62:21–8.

[25] Azhar S, Reaven E. Scavenger receptor class BI and selective cholesteryl ester uptake: partners in the regulation of steroidogenesis. Mol Cell Endocrinol 2002;195:1–26.
[26] Schlorff EC, Hussain K, Somani SM. Dose and time dependent effects of ethanol on antioxidant system in rat testis. Alcohol 1999; 18:203–14.
[27] Medicherla R, Leers-Sucheta S, Luo Y, Azhar S. Impaired activation of AP-1 and altered expression of constituent proteins in rat adrenal during ageing. Mech Ageing Dev 2001;122:1169–86.
[28] Medicherla R, Leers-Sucheta S, Luo Y, Azhar S. Age-dependent modulation of NF-κB expression in rat adrenal gland. Mech Ageing Dev 2002;123:1211–27.
[29] Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochim Biophys Acta 1991;1072: 129–57.
[30] Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 1999;39:67–101.
[31] Wisdom R. AP-1: One switch for many signals. Exp Cell Res 1999; 253:180–85.
[32] Silverman N, Maniatis T. NF-κB signaling pathways in mammalian and insect innate immunity. Genes Dev 2001;15:2321–42.
[33] Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell 2002; 109:S81–96.
[34] Kitabchi AE, Williams RH. Adrenal gland in vitamin E deficiency: lipid peroxidation and malonaldehyde production in vitro. J Biol Chem 1968;243:3248–54.
[35] Burczynski JM, Southard SJ, Hayes JR, Longhurst PA, Colby HD. Changes in mitochondrial and microsomal lipid peroxidation and fatty acid profiles in adrenal glands, testes and livers from alpha-tocopherol-deficient rats. Free Radic Biol Med 2001;30:1029–39.
[36] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13:1145–55.
[37] Wang X, Quinn PJ. Vitamin E and its function in membranes. Prog Lipid Res 1999;38:309–36.
[38] Xanthoudakis S, Smeyne RJ, Wallace JD, Curran T. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc Natl Acad Sci USA 1996;93:8919–23.
[39] Rothwell DG, Barzilay G, Gorman M, Morera S, Freemont P, Hickson ID. The structure and functions of the HAP1/Ref-1 protein. Oncol Res 1997;9:275–80.
[40] Martindale JL, Holbrook N. Cellular response to oxidative stress: signaling for suicide and survival. Cell Physiol 2002;192:1–15.
[41] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
[42] Haberland A, Damerau W, Stosser R, Schimke I, Baumann G. Fe^{2+}/vitamin C—an appropriate *in vitro* model system to initiate lipid peroxidation. J Inorg Biochem 1996;61:43–53.
[43] Kulkarni AP, Hodgson E. A comparison of NADPH and cumene hydroperoxide-stimulated lipid peroxidation in mouse hepatic microsomes. Int J Biochem 1981;13:811–6.
[44] Pyles LA, Stejskal EJ, Einzig S. Spectrophotometric measurement of plasma 2-thiobarbituric acid-reactive substances in the presence of hemoglobin and bilirubin interference. Proc Soc Exp Biol Med 1993; 202:407–19.
[45] Markwell MAK, Hass SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978;87:206–10.
[46] Peterson GL. A simplification of the protein assay method of Lowry *et al* which is more generally applicable. Anal Biochem 1977;83: 346–56.
[47] Pennypacker KR, Hong J-S, Douglass J, McMillian MK. Constitutive expression of AP-1 transcription factors in the rat adrenal: effects of nicotine. J Biol Chem 1992;267:20148–52.
[48] Evans AR, Limp-Foster M, Kelley MR. Going over ref-1. Mutat Res 2000;461:83–108.
[49] Flaherty DM, Monick MM, Hunninghake GW. AP endonucleases and the many functions of Ref-1. Am J Respir Cell Mol Biol 2001; 25:664–7.
[50] Quinn PG, Payne AH. Oxygen-mediated damage to microsomal cytochrome P-450 in cultured Leydig cells: role in steroidogenic desensitization. J Biol Chem 1984;259:4130–5.
[51] Quinn PG, Payne AH. Steroid product-induced, oxygen-mediated damage of the microsomal cytochrome P-450 enzymes in Leydig cell cultures: relationship to desensitization. J Biol Chem 1985;260:2092–9.
[52] Cheng B, Kowal J. Analysis of adrenal cholesteryl esters by reversed phase high performance liquid chromatography. J Lipid Res 1994; 35:1115–21.
[53] Coniglio JG. Testicular lipids. Prog Lipid Res 1994;33:387–401.
[54] Barker CW, Fagan JB, Pasco DS. Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress. J Biol Chem 1994;269:3985–90.
[55] Morel Y, Barouki R. Down-regulation of cytochrome P450 1A1 gene promoter by oxidative stress: critical contribution of nuclear factor 1. J Biol Chem 1998;273:26969–76.
[56] Luo L, Chen H, Zirkin B. Leydig cell aging: steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme. J Androl 2001;22:149–56.
[57] Fung H, Kow YW, van Houten B, Taatjes DJ, Hatahet Z, Janssen YM, Vacek P, Faux SP, Mossman BT. Asbestos increases mammalian AP-endonuclease gene expression, protein levels, and enzyme cativity in mesothelial cells. Cancer Res 1998;58:189–94.
[58] Tell G, Crivellanto E, Pines A, Paron I, Pucillo C, Manzini G, Bandiera A, Kelley KR, Loreto CD, Damante G. Mitochondrial localization of APE/Ref-1 in thyroid cells. Mutat Res 2001;485:143–52.
[59] Asai T, Kambe F, Kikumori T, Seo H. Increase in Ref-1 mRNA and protein by throtropin in rat thyroid FRTL-5 cells. Biochem Biophys Res Commun 1997;236:71–4.
[60] Suzuki S, Nagaya T, Suganuma N, Tomoda Y, Seo H. Induction of immediate early genes (IEGS) and ref-1 by human chrionic gonadotropin in murine leydig cell line (MA-10). Biochem Mol Biol Int 1998;44:217–24.