Pharmacia Biosensor, Uppsala, Sweden) permits the monitoring of macromolecular interactions in real time using a surface plasmon resonance (SPR) sensor (*28*). The running buffer used for immobilization and the binding assay consisted of 25 mM Tricine, 160 mM KCl, 5 mM $MgCl_2$, and 0.05% Tween 20 (pH 7.8). Before immobilization of biotinylated ERE, the surface of the SA (streptavidin-treated) sensor chip was washed with five 5 μL injections of 100 mM NaOH and 50 mM HCl each with a constant flow of running buffer of 20 μL/min. For denaturation, oligonucleotides were heated at 105 °C for 5 min and then chilled on ice before immobilization. 5′-End-biotinylated single-stranded oligonucleotides (Human *pS2* ERE) diluted with running buffer were immobilized to a flow cell (Fc2) of an SA sensor chip at about 200 RU by serial 5 μL injection with a constant flow of running buffer of 5 μL/min. Then, the sensor chip surface was blocked by biotin, followed by five washes with NaOH and HCl. The complementary oligonucleotide was annealed to the immobilized ERE by a 10 μL injection.

Analysis of ER−ERE Binding. Incubating at 37 °C for 5 min liganded human ERα and ERβ (2×10^{-7} M) with 10^{-7} M E_2 or 10^{-5} M isoflavones and their metabolites. Then, the liganded ER was introduced by a 40 μL injection over the surfaces coated with double-stranded ERE via a sample loop. Each binding cycle was performed with a constant flow of buffer of 20 μL/min at 25 °C. ER protein was injected during the "binding" phase, and running buffer was injected across the flow cells during the "dissociating" phase for 120 s. As significant amounts of ER were still bound to the ERE at the end of the injection, the complementary oligonucleotide was removed with a 5 μL injection of 100 mM NaOH and 50 mM HCl, each with a constant flow of running buffer of 20 μL/min for regeneration. Data were collected as the subtracted curve (Fc1 − Fc2) at 1 Hz. The binding activity of liganded ER to ERE was expressed as percent activity, that is, binding response with 100 nM E_2 as 100% and that without chemical (DMSO, 0.1%) as 0%. All samples contained 0.1% DMSO.

Measurement of 8-OxodG in DNA from Cultured Human Mammary Epithelial Cells Treated with Genistein, Daidzein, and Their Metabolites. Human mammary epithelial cells (MCF-10A cells and MCF-7 cells) were trypsinized, and 5×10^5 cells were plated into a 10 cm diameter dish with seeding medium. Cells were allowed to attach and grow until 70−90% confluency for 3−4 days. Then, cells were treated with isoflavones at 37 °C for 1 h and trypsinized and washed three times with cold PBS. Under anaerobic conditions, DNA was extracted using lysis buffer, RNase A, and proteinase K. After ethanol precipitation, DNA was digested to component nucleosides with nuclease P_1 and bacterial alkaline phosphatase and then analyzed by HPLC-ECD as previously described (*29*). In certain experiments, breast cancer MCF-7 cells were pretreated with an inhibitor of GSH biosynthesis (BSO, 100 μM, 18 h) to decrease GSH levels to that of normal mammary cells.

Measurement of GSH Content in MCF-10A and MCF-7 Cells. Cells were washed twice with PBS, followed by addition of 100 μL/10^6 cells of 5% (w/v) trichloroacetic acid to precipitate proteins. Then, cells were homogenized for 5 s with a microhomogenizer with a Teflon-coated pestle and centrifuged at 18500*g* for 10 min at 4 °C. The supernatant was diluted with 0.1 N HCl, and levels of GSH were quantitated with an HPLC-ECD using a gold electrode (Eicom, Kyoto, Japan), as described previously (*30*).

Analysis of 8-OxodG Formation in Calf Thymus DNA by Genistein, Daidzein, and Their Metabolites in the Presence of NADH and Cu(II). DNA fragments (100 μM per base) from calf thymus were incubated with isoflavones, Cu(II), and NADH at 37 °C for the indicated times. DNA fragments were denatured by heating at 90 °C for 5 min, followed by chilling on ice before incubation. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and then analyzed by HPLC-ECD, as described previously (*29*).

Preparation of ^{32}P-5′-End-Labeled DNA Fragments. Exon-containing DNA fragments obtained from the human *p53* tumor suppressor gene (*31*) were prepared as described previously (*32*). A 5′-end-labeled 650 bp fragment (*Hin*dIII* 13972−*Eco*RI* 14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (*, ^{32}P-label). The 650 bp fragment was further digested with *Apa*I to obtain a singly labeled 443 bp fragment (*Apa*I 14179−*Eco* RI* 14621) and a 211 bp fragment (*Hin*dIII* 13972−*Apa*I 14182). The fragment was prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 protooncogene (*33*). A singly labeled 98 bp fragment (*Ava*I* 2247−*Pst*I 2344) was obtained according to the method described previously (*34*). Nucleotide numbering starts with the *Bam*HI site (*33*).

Detection of DNA Damage by Genistein, Daidzein, and Their Metabolites in the Presence of NADH and Cu(II). A standard reaction mixture (in a 1.5 mL Eppendorf microtube) contained the isoflavones, Cu(II), NADH, ^{32}P-5′-end-labeled DNA fragments, and calf thymus DNA (5−10 μM per base) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37 °C for the indicated times, the DNA fragments were heated at 90 °C in 1 M piperidine for 20 min, where indicated, and treated as described previously (*35*). In certain experiments, the DNA was treated with 6 units of Fpg protein in 10 μL of reaction buffer [10 mM HEPES−KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/mL BSA] at 37 °C for 2 h. The preferred cleavage sites were determined by direct comparison of the labeled, cleaved oligonucleotides with a standard 5′-end-labeled Maxam−Gilbert sequencing reaction (*36*) (LKB 2010 Macrophor, LKB Pharmacia Biotechnology Inc.). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL, LKB Pharmacia Biotechnology Inc.).

RESULTS

Cell Proliferative Activity of Genistein and Daidzein in MCF-7 Cells. The effects of isoflavones and their metabolites on cell proliferation were measured by an E-screen assay. Genistein induced maximal proliferative activity at 10^{-6} M ($P < 0.01$), with significant differences relative to solvent control (0.1% DMSO) starting at 10^{-7} M ($P < 0.05$) (Figure 2A). The intensity of maximal estrogenic activity of genistein was about 90% of estradiol. Orobol showed a significant proliferative activity at 10^{-5} M ($P < 0.05$). Daidzein showed

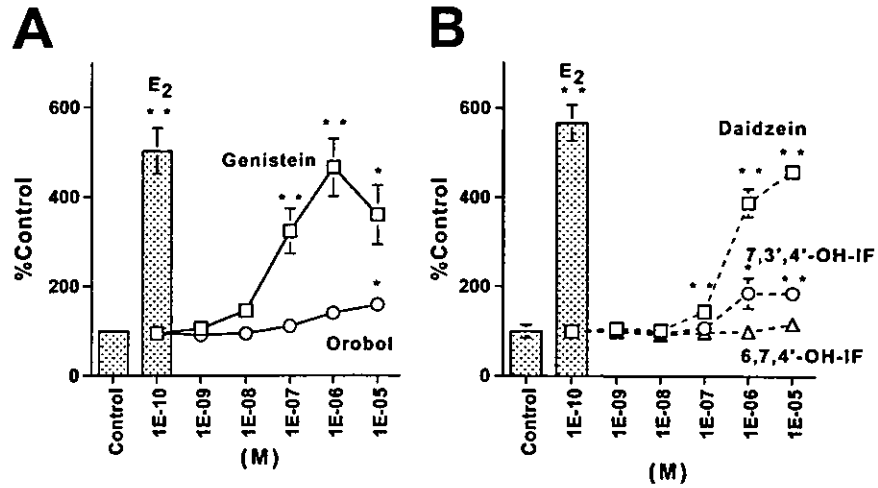

FIGURE 2: Relative estrogenic activities of isoflavones and their metabolites. MCF-7 cells were incubated with genistein, orobol (A), daidzein, 7,3′,4′-OH-IF, or 6,7,4′-OH-IF (B) at 37 °C for 6 days. Cells were trypsinized, harvested, and then counted. Results are expressed as means and SE of values obtained from six to nine independent experiments. Key: *, $P < 0.05$, and **, $P < 0.01$; significant difference compared with the control by Student's *t*-test.

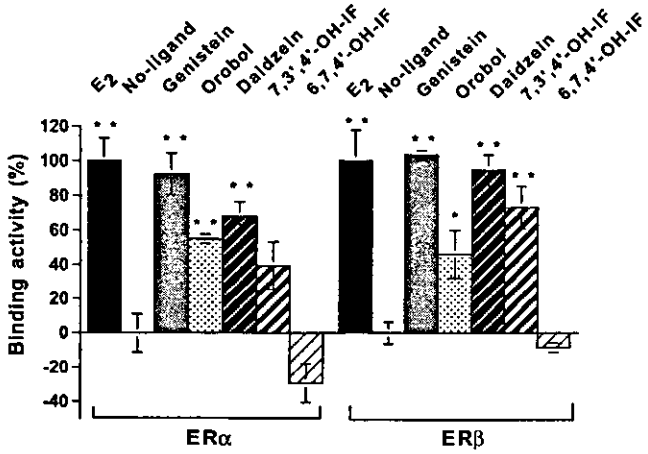

FIGURE 3: ER−ERE binding activities in the presence of isoflavones and their metabolites. Human ERα and ERβ (20 nM) were liganded with 100 nM E_2 or 10 μM phytoestrogens by incubation at 37 °C for 5 min. Then, the liganded ER was introduced by a 40 μL injection over the sensor chip surface immobilized with double-stranded human *pS2* ERE. The binding activity of liganded ER to ERE was expressed as percent activity, that is, binding response with 100 nM E_2 as 100% and no ligand (DMSO, 0.1%) as 0%. Results are expressed as means and SE of percent activity obtained from three independent experiments. Key: *, $P < 0.05$, and **, $P < 0.01$; significant difference compared with the no ligand condition by Student's *t*-test.

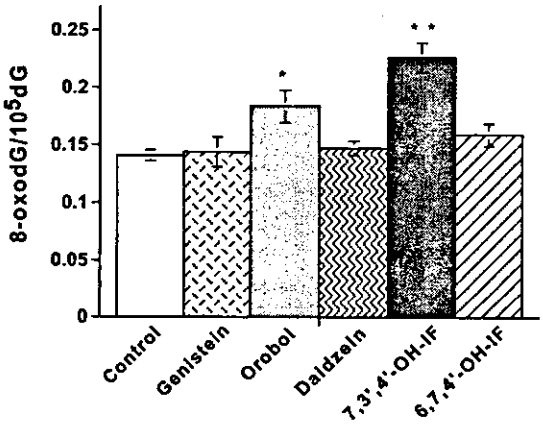

FIGURE 4: Intracellular 8-oxodG formation by isoflavone metabolites in MCF-10A cells. MCF-10A cells were treated with 10 μM isoflavones or their metabolites in the experimental medium at 37 °C for 1 h. Results are expressed as means and SE of values obtained from three independent experiments. Key: *, $P < 0.05$, and **, $P < 0.01$; significant difference compared with the control by Student's *t*-test.

maximal proliferative activity at 10^{-5} M ($P < 0.01$), with significant differences relative to controls starting at 10^{-7} M ($P < 0.05$) (Figure 2B). Daidzein exhibited an estrogenicity about 80% that of estradiol. 7,3′,4′-OH-IF showed significant proliferative activity at 10^{-6} and 10^{-5} M. 6,7,4′-OH-IF had no significant proliferative effect.

Binding of Isoflavone-Liganded ERα and ERβ to ERE. The binding activity of liganded ER to ERE was measured by using an SPR sensor (Figure 3). Genistein- and daidzein-liganded ER had significantly elevated binding activity, as did E_2. The binding activity of liganded ERβ was slightly higher than that of ERα. Binding activity was also detected for the metabolites, orobol and 7,3′,4′-OH-IF, but it was lower than in the parent isoflavones. On the other hand, 6,7,4′-OH-IF attenuated binding to ERE, although there was no significant difference relative to nonliganded ER. Similar results were obtained with ERα and ERβ.

Induction of 8-OxodG Formation in Human Cultured Mammary Cells Treated with Isoflavone Metabolites. Orobol and 7,3′,4′-OH-IF significantly increased 8-oxodG formation in normal mammary epithelial MCF-10A cells, but no significant increase was observed in cells treated with genistein, daidzein, or 6,7,4′-OH-IF (Figure 4). In contrast, there was no significant increase in 8-oxodG formation in MCF-7 breast cancer cells treated with isoflavones and their metabolites compared to controls (data not shown). The GSH level in MCF-7 cells was 2-fold higher than in MCF-10A cells (data not shown). The GSH level decreased to 50% in MCF-7 cells following pretreatment with BSO, and thereafter, significant increases were observed in MCF-7 cells treated with orobol and 7,3′,4′-OH-IF (data not shown).

Formation of 8-OxodG in Calf Thymus DNA by Isoflavone Metabolites in the Presence of Cu(II) and NADH. Cu(II)-mediated 8-oxodG formation in calf thymus DNA treated with isoflavones, in the presence and absence of NADH, was examined using HPLC-ECD (Figure 5). In the case of

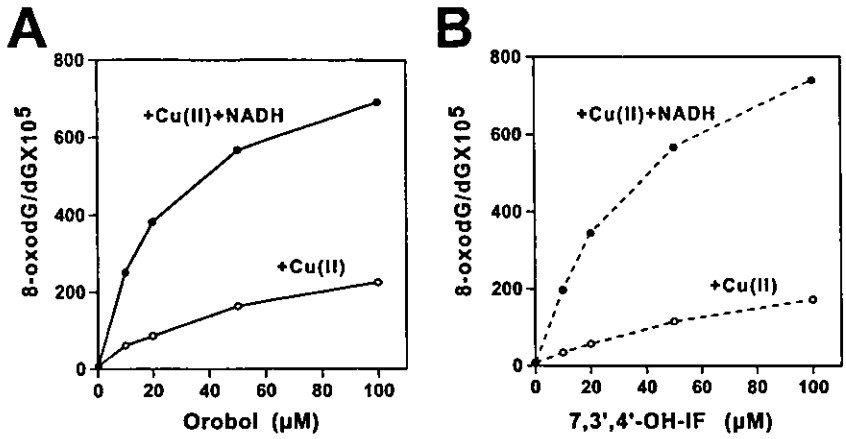

FIGURE 5: Formation of 8-oxodG in calf thymus DNA treated with isoflavone metabolites in the presence of Cu(II) and NADH. The reaction mixture contained 100 μM/base calf thymus DNA, the indicated concentrations of orobol (A) or 7,3′,4′-OH-IF (B), 20 μM $CuCl_2$, and no or 200 μM NADH in 4 mM phosphate buffer (pH 7.8) containing 1 μM DTPA. After incubation at 37 °C for 1 h, 0.1 mM DTPA was added to stop the reaction, and DNA was precipitated in ethanol and enzymatically digested into nucleosides. Then, the levels of 8-oxodG were quantitated by HPLC-ECD.

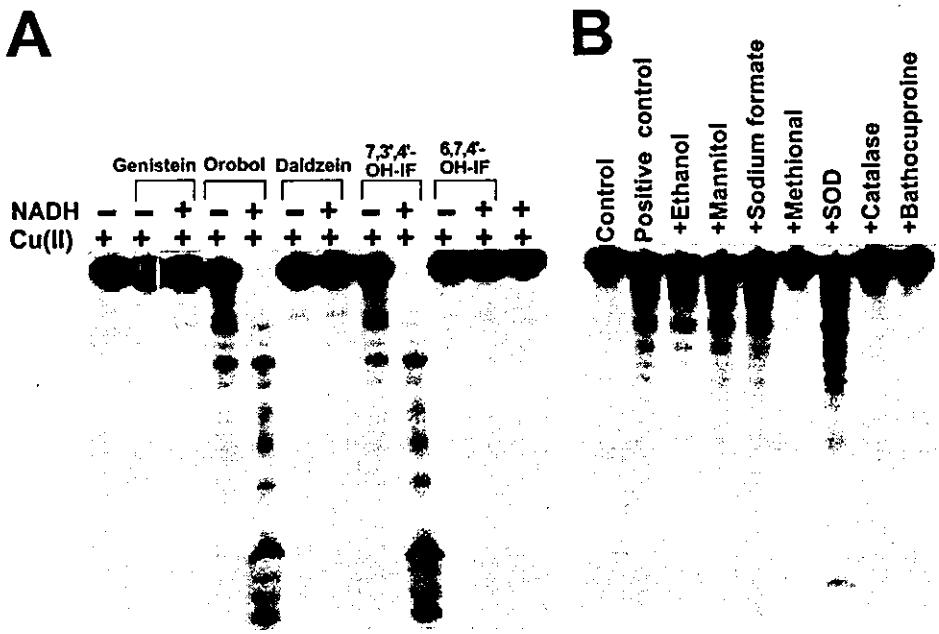

FIGURE 6: Autoradiogram of ^{32}P-labeled DNA fragments incubated with isoflavones in the presence of Cu(II) and NADH and the effects of scavengers. (A) The reaction mixture contained the ^{32}P-5′-end-labeled 261 bp DNA fragment, 20 μM/base thymus DNA, 100 μM isoflavones, 20 μM $CuCl_2$, and 200 μM NADH in 10 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. (B) The reaction mixtures contained the ^{32}P-5′-end-labeled 211 bp DNA fragment, 20 μM/base sonicated calf thymus DNA, 100 μM 7,3′,4′-OH-IF, and 20 μM $CuCl_2$ in 10 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Scavenger or bathocuproine was added as follows: 5% ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, 150 units/mL SOD, 150 units/mL catalase, and 50 μM bathocuproine. The mixtures were incubated for 1 h at 37 °C. The DNA fragments were treated with 1 M piperidine for 20 min at 90 °C and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel.

genistein, daidzein, and 6,7,4′-OH-IF, no increase in 8-oxodG formation was observed, even in the presence of NADH and Cu(II) (data not shown). Conversely, the level of 8-oxodG increased in parallel with concentrations of orobol (Figure 5A) and 7,3′,4′-OH-IF (Figure 5B) in the presence of Cu(II). When a physiological concentration of NADH (200 μM) (*37*) was added, 2−3-fold increases in 8-oxodG formation were observed. The metabolites with 3′- and 4′-positions of the hydroxy group in the B ring of the isoflavone structure caused oxidative DNA damage.

Damage to ^{32}P-Labeled DNA Fragments by Isoflavone Metabolites in the Presence of Cu(II) and NADH. Figure 6A shows an autoradiogram of DNA fragments treated with isoflavones and their metabolites in the presence and absence of Cu(II) and NADH. Oligonucleotides were detected on the autoradiogram following DNA cleavage. Genistein, daidzein, and 6,7,4′-OH-IF caused no DNA damage, even in the presence of Cu(II) and NADH. Orobol and 7,3′,4′-OH-IF caused DNA damage in the presence of Cu(II). The intensity of DNA damage increased with successive concentrations of the metabolites (data not shown). When NADH was added, Cu(II)-mediated DNA damage was enhanced. When Fe(III)EDTA was used in place of Cu(II), slight DNA damage was observed (data not shown), indicating the crucial role of metal ions. The catechol-type metabolites caused Cu(II)-mediated oxidative DNA damage, whereas genistein and daidzein did not, even in the presence of Cu(II) and NADH, as seen in the measurement of 8-oxodG content.

Effects of Scavengers and Bathocuproine on DNA Damage by Isoflavone Metabolites. Figure 6B shows the effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage induced by 7,3′,4′-OH-IF in the presence of Cu(II). Inhibition of DNA damage by catalase and bathocuproine suggests the involvement of hydrogen peroxide (H_2O_2) and Cu(I). Methional reduced the amount of DNA damage, although other typical hydroxyl radical (•OH) scavengers, ethanol, mannitol, and sodium formate did not decrease damage. Furthermore, SOD had no effect on DNA damage. In the case of the genistein metabolite orobol, similar effects of scavengers were observed (data not shown).

Site Specificity of DNA Cleavage by Isoflavone Metabolites. Isoflavone metabolites/Cu(II) caused little strand breakage, as detected without treatment (Figure 7A, lane 2, and Figure 7E). In addition, an increase in the number of oligonucleotides following piperidine treatment suggested that metabolites caused base modification/ liberation (Figure 7A, lane 4). Fpg treatment increased oligonucleotides, indicating the formation of 8-oxoG and other oxidized bases (Figure 7A, lane 6). To examine site specificity, an autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in the human c-Ha-*ras*-1 protooncogene and *p53* tumor suppressor gene. Orobol/Cu(II)/NADH induced piperidine-labile sites preferentially at thymine residues and Fpg-sensitive sites at guanine residues in the 5′-TG-3′ sequence of the c-Ha-*ras*-1 gene (Figure 7B,C). Fpg treatment induced significant cleavage of the guanine residue of the ACG sequence complementary to codon 273, a well-known hot spot (*38*, *39*) in the *p53* gene (Figure 7G). Piperidine treatment cleaved cytosine and guanine residues at the ACG (Figure 7F) to some extent. Similar results were obtained with 7,3′,4′-OH-IF (data not shown).

DISCUSSION

The present study showed that genistein and daidzein exerted cell proliferative activity on estrogen-sensitive MCF-7 cells, as reported previously (*40*, *41*), while their metabolites had little or no activity. In accordance with the data on cell proliferation, the SPR sensor showed that genistein and daidzein induced higher affinity binding of ER to ERE, while the metabolites had little or no binding activity. Although Cheskis et al. (*42*) showed that SPR was available for a binding assay of estrogen-liganded ER−ERE, we further demonstrated that an SPR sensor could estimate the potency of environmental estrogens. Genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-trihydroxyisoflavone) have similar chemical structures to endogenous estrogens. Their structural similarity to estrogens permits binding with ER (*27*). The 4′-hydroxy position on the B ring and its spatial orientation relative to the 7-hydroxy group on ring A are primarily responsible for the estrogenic activity of flavonoids (*43*). Excessive hydroxyl groups in ring B at the 3′-position, as in orobol (5,7,3′,4′-tetrahydroxyisoflavone) and 7,3′,4′-OH-IF, is thought to attenuate the binding of ER and ERE. A hydroxyl group in ring A at the 6-position, as in 6,7,4′-OH-IF, shows a similar effect. Our results further supported that isoflavones such as genistein and daidzein may induce cell proliferation through ER−ERE binding. Kuiper et al. (*44*) showed that the relative binding affinity of genistein to ERβ was significantly higher (about 20-fold) than ERα, whereas Nikov et al. (*45*) demonstrated slightly higher affinity between ERE and isoflavone-saturated ERβ than with ER α, using fluorescence polarization. The latter data are consistent with our results using an SPR biosensor that showed slightly higher affinity of isoflavone-liganded ERβ than ERα to ERE. The results from the E-screen assay and binding assay were almost coincident with little discrepancy. Orobol and 7,3′,4′-OH-IF bound relatively efficiently to both estrogen receptors but only poorly stimulated cell proliferation in MCF-7 cells. This may be explained by the difference between physicochemical responses and biological systems with a threshold range. In addition, Wong et al. (*46*) have reported that nuclear receptor ligands can be functionally selective and may differentially affect the interaction of receptors with coactivators and/or corepressors to initiate transcription. Endogenous estrogens cause cancer by stimulating cell proliferation through ER−ERE binding (*47*, *48*). Similarly, these estrogen-like substances can be mitogenic in estrogen-sensitive tissues such as uterus and breast, which may contribute to tumor promotion.

Van Duursen et al. (*49*) demonstrated that constitutive CYP1A1 activity was very low in both MCF-7 and MCF-10A cells. In addition, Price et al. (*50*) showed that the optimal time for induction of metabolism by a CYP1A inducer was 72 h in rat hepatocytes. Therefore, metabolism in cultured cells was negligible under our experimental design (1 h incubation). This may explain why genistein and daidzein themselves did not induce DNA damage. On the other hand, the addition of the isoflavone metabolites, orobol and 7,3′,4′-OH-IF, caused oxidative DNA damage. Possible mechanisms of oxidative DNA damage mediated by metabolites of genistein and daidzein can be envisioned on the basis of our results. Dihydroxy forms of the isoflavone metabolites, orobol and 7,3′,4′-OH-IF, can be autoxidized to semiquinone radicals and further to quinone forms. Generation of $O_2^{\bullet -}$ would then occur, coupled with the autoxidation of metabolites. Thereafter, $O_2^{\bullet -}$ is dismutated to generate H_2O_2. In the presence of metal ions, H_2O_2 causes oxidative DNA damage. Inhibitory effects of catalase and bathocuproine on DNA damage by the metabolites suggest that H_2O_2 and Cu(I) participate in DNA damage. Although typical •OH scavengers showed no inhibitory effects on DNA damage, methional attenuated DNA damage, suggesting the involvement of reactive species such as Cu(I)−hydroperoxo complexes that are less reactive than •OH (*51*). The addition of NADH efficiently enhanced oxidative DNA damage by isoflavone metabolites. This can be explained by our results and those of our previous studies (*21*, *52*, *53*). NADH reduces quinone forms and semiquinone radicals to dihydroxy forms, resulting in enhanced generation of reactive oxygen species and DNA damage through the redox cycle. It is interesting that Fpg and piperidine treatment revealed that orobol and 7,3′,4′-OH-IF can affect the cytosine and guanine of the ACG sequence complementary to codon 273, a hot spot in the *p53* gene.

Oxidative DNA damage plays an important role in carcinogenesis (*54*). Orobol and 7,3′,4′-OH-IF significantly induced 8-oxodG formation in MCF-10A cells, and in MCF-7 cells with GSH levels decreased to 50% with BSO treatment. Normal human breast tissue has a lower GSH content than breast tumor (*55*). Therefore, the depletion of GSH by BSO in MCF-7 cells may provide an insight into

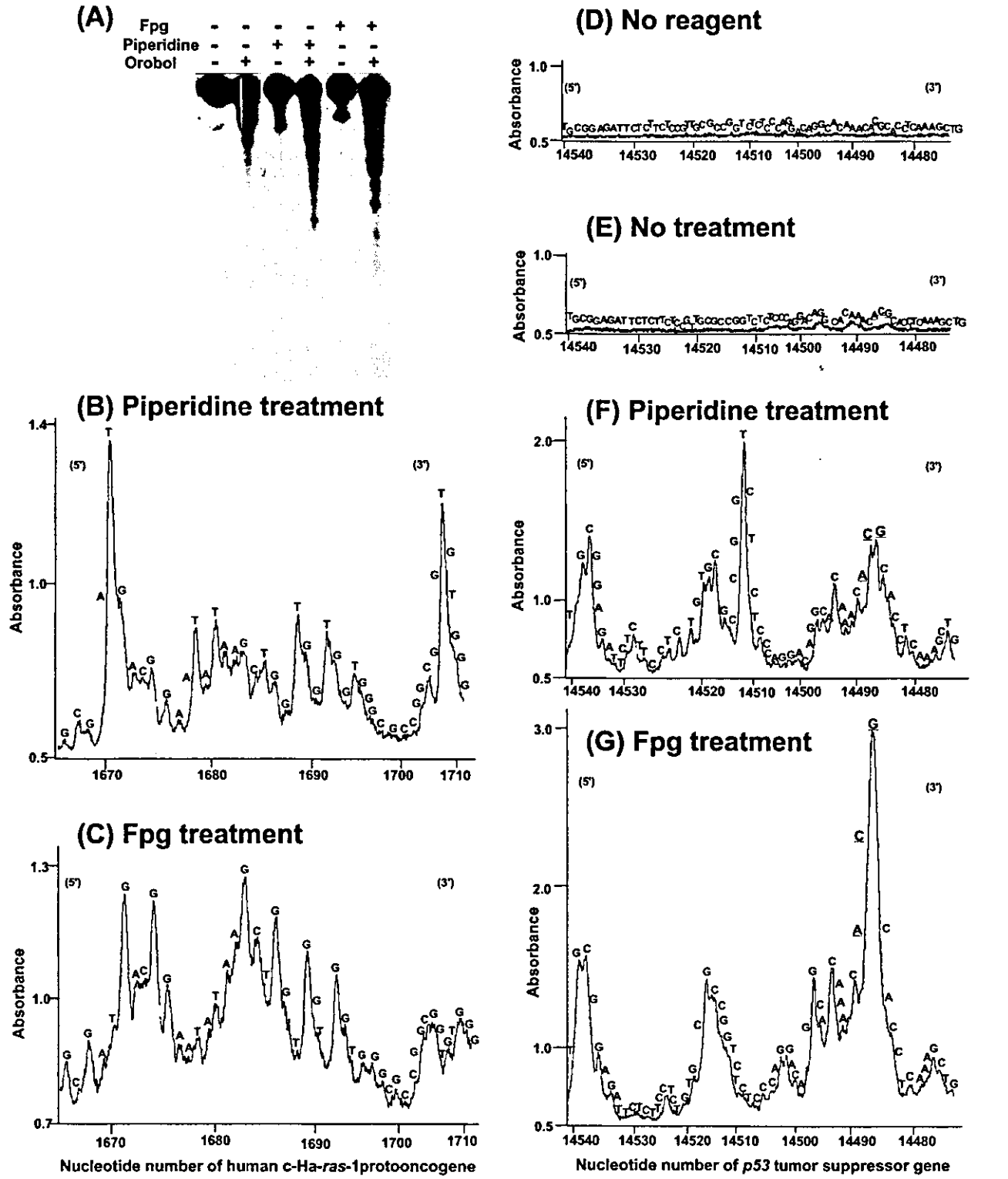

FIGURE 7: Site specificity of DNA cleavage induced by isoflavone metabolites in the presence of Cu(II) and NADH. The reaction mixture contained the ^{32}P-5′-end-labeled 261 bp (*Ava*I* 1645−*Xba*I 1905) (B, C) or 443 bp (*Apa*I 14179−*Eco*RI* 14621)(A, D−G) DNA fragment, 20 μM/base calf thymus DNA, 5 μM orobol, 20 μM $CuCl_2$, and 200 μM NADH in 10 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation for 1 h at 37 °C, the DNA fragments were treated with piperidine (A, B, D, F) or Fpg protein (A, C, G) and electrophoresed by the method described in Materials and Methods. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (B−G). The horizontal axis shows the nucleotide number of the c-Ha-*ras*-1 protooncogene (B, C) and the human *p53* tumor suppressor gene (D−G), and underscoring shows the complementary sequence to codon 273 (nucleotide numbers 14486−14488).

normal breast tissue condition. Several studies showed increased formation of 8-oxodG in breast cancer tissue from patients relative to noncancerous breast tissue from controls (*56*, *57*), suggesting that accumulation of 8-oxodG in DNA is a major contributor to breast carcinogenesis. The present results and literature thus indicate that oxidative DNA damage by isoflavone metabolites plays a role in tumor initiation and cell proliferation by isoflavones via ER−ERE

binding induces tumor promotion and/or progression, resulting in cancer of estrogen-sensitive organs. Our study raises the possibility that genistein and daidzein are carcinogenic in estrogen-sensitive organs, even though isoflavones are generally regarded as chemopreventive agents.

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